

INVESTIGATIONS INTO OBESITY USING
ANTHROPOMETRIC, SERUM AND
GENETIC MARKERS

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**Investigations into obesity using anthropometric, serum and
genetic markers.**

by

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A thesis submitted to the School of Graduate Studies in partial
fulfilment of the requirements for the degree of Master of Science

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May, 2010

St. John's

Newfoundland

Abstract

Obesity is one of today's most visible public health problems with worldwide incidence at over a billion people. To understand such a vast problem requires one to study the issue from different angles. This includes studying how obesity is measured, and how metabolic and genetic factors influence the obesity phenotype. This thesis investigates the accuracy of the body mass index, a trusted measurement tool, compared to an industry standard measuring tool DXA. It also investigates the effects of serum calcium on serum lipid levels and how polymorphisms in two genes associated with the storage of fat in the human adipocyte affect obesity phenotypes.

Although body mass index (BMI) is the most widely used measure of obesity, debate still exists on how accurately BMI defines obesity. In this study, adiposity status defined by BMI and DXA were compared in a large population to evaluate the accuracy of BMI. A total of 1691 adult volunteers from Newfoundland and Labrador (NL) participated in the study. BMI and %BF were measured for all subjects following a 12 hour fasting period. Subjects were categorized as underweight (UW), normal weight (NW), overweight (OW), or obese (OB) based on BMI and %BF criteria. Differences between the two methods were compared within gender and by age groups. According to BMI criteria 1.2% of women were classified as UW, 44.4% as NW, 34.1% as OW and 20.3% as OB. When women were classified according to %BF criteria 2.2% were UW, 29.7% were NW, 31.0% were OW and 37.1% were OB. The overall discrepancy between the two methods for women was substantial at 34.8% (14.6 % for NW and 16.9% for OB, $p < 0.001$). In men the overall discrepancy was 35.2% between BMI and DXA (17.6% for

OW and 13.5% for OB, $p < 0.001$). Misclassification by BMI was dependent on age, gender and adiposity status. In conclusion, BMI misclassified adiposity status in approximately one third of women and men compared with DXA. Caution should be taken when BMI is used in clinical and scientific research as well as clinical practice.

Some epidemiological evidence shows a link between abnormality of lipid profiles and variations in serum calcium. However, it is unknown whether this result was influenced by confounding factors. The present study was designed to investigate the relationship between serum lipids and calcium. Serum calcium was corrected for albumin. Major confounding factors including age, gender, medications, menopause, parathyroid hormone (PTH) and 25-OH-vitamin D status were controlled in analyses. A total of 1907 adult subjects from the province of Newfoundland and Labrador (NL), Canada participated in the study. Significant positive correlations were detected between serum total cholesterol and high density lipoprotein-cholesterol (HDL-c) with variations of serum Ca^{++} in both genders ($p < 0.05$ -0.0001). Significant positive correlations were additionally detected between triglycerides (TAG) and low density lipoprotein-cholesterol (LDL-c) with Ca^{++} in women only ($p < 0.0001$) in partial correlation analyses. Similar significant results were detected in both females and males not taking any medication. Analyses were performed based on menopausal status as well. Significant correlations were seen in both pre- and post-menopausal women but higher correlation coefficients were observed in pre-menopausal women as compared to post-menopausal women. Subjects with low calcium levels had the lowest concentration of total cholesterol, TAG, HDL-c and LDL-c, while subjects with high calcium levels had the

~~highest concentration of all four markers in women. The significant associations between~~
cholesterol, TAG and LDL-c and serum Ca^{++} remained after calcium was adjusted for 25-OH-vitamin D and PTH. Our results indicate that the abnormality of serum lipid profiles are significantly correlated with altered serum Ca^{++} levels independent of age, obesity status, medication, phosphorus, magnesium, 25-OH-vitamin D and PTH.

Perilipin (PLIN) is the major component of lipid droplet coating proteins, which play a key role in regulating human adipocyte triglyceride metabolism. Adipophilin is another lipid droplet coating protein that has been implicated to be involved in subcellular lipid trafficking. The association of perilipin and adipophilin with lipid storage droplets and lipid trafficking makes them potential candidate genes for obesity and lipid abnormality.

This study investigated whether single nucleotide polymorphisms (SNP) in the PLIN and ADFP genes are associated with variations in body composition and serum lipid abnormalities. A total of 1269 healthy subjects (1002 females, 267 males) were recruited from the genetically homogeneous population of Newfoundland and Labrador (NL), Canada. All subjects were at a minimum third generation residents of NL, between the ages of 19-62. Percentages body fat (%FAT) was measured using DXA. Fasting serum concentrations of glucose, cholesterol, triacylglyceride (TAG), HDL-C, LDL-C, and insulin were measured and the risk ratio for cardiovascular disease (cholesterol/HDL-C) was calculated. Genomic DNA was extracted from whole blood. All statistical analyses were performed using the statistical software R or SPSS software version 16 for

Windows; with significance set at $p < 0.05$. Hardy-Weinberg equilibrium was tested using chi-square analysis.

Three SNPs located within the PLIN coding region were selected. SNP:rs4932241, an A/C SNP located in the 3' untranslated region, SNP:rs2289487, an A/G SNP located between the 2nd and 3rd exon, and SNP:rs894160, an A/G SNP located between the 6th and 7th exon. In ADFP, two A/G polymorphisms were selected. SNP:rs3824369 located in the 5' intronic region and SNP:rs35629534 located in the 3' untranslated region. Genotyping was performed using TaqMan® validated SNP genotyping assays from Applied Biosystems on an ABI 7000 Sequence Detection System. All SNPs investigated in this study were in Hardy Weinberg equilibrium.

The results showed that there were no significant associations within the PLIN SNPs for body composition or serum lipid parameters in our population. The results of the present study do not support a significant role for genetic variations within the perilipin gene in the regulation of body composition and serum lipid profiles in the NL population.

The results revealed a significant association between ADFP SNP:rs3824369 and %BF, serum cholesterol and serum LDL-c after controlling for the effects of gender and age. Carriers of the major allele of SNP:rs3824369 had significantly higher fasting levels of LDL-c and significantly higher fasting levels of serum cholesterol compared to homozygote carriers of the minor allele. In addition, carriers of the major allele of SNP:

~~rs3824369 had significantly higher %BF compared to homozygote carriers of the minor~~
allele. To our knowledge this is the first report of an association between a SNP in *ADFP*
and variations in body fat or serum lipids.

Acknowledgements

I would like to express my genuine appreciation and thanks to everybody who supported me during my graduate program.

I would like to start by recognizing the help and dedication of my supervisor, Dr. Guang Sun. Throughout my graduate career he has supported me in all of my decisions and was always available when I needed help or encouragement. His dedication to his graduate students is evident by the work they produce and he provides a great example of how we should act when we are finally in his position.

I would like to thank all the members of the Sun lab including current and past members but most importantly Glynn Martin for always having the right answer, Jennifer Shea for her support with editing and judgement, and Hongwei Zhang for her immense dedication to work in the lab and for always being pleasant and helpful when I needed it.

I would like to acknowledge Deborah Quinlan for all her help and support throughout my graduate career.

I would like to thank my wife for putting up with me and for constantly accepting my excuses throughout the years.

I would also like to thank my parents for their support and encouragement throughout my academic career.

I dedicate this dissertation to my wife, Dr. Kristi Kennedy, and my children, Jack and Maddison for always showing me unconditional love every day. Thinking of coming home to you makes every day a great day.

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1.1: Obesity in today's society.

Obesity is one of today's most visible public health problems with worldwide incidence at over a billion people [1]. The prevalence of overweight and obesity is increasing at an alarming rate in both the developed and the developing world. This has resulted in increased public health challenges worldwide. Thirty three percent (1.3 billion people) of the worlds adult population was overweight or obese in 2005 with estimates of up to 58% of the adult population (3.3 billion) either overweight or obese by the year 2030 [2]. Although a larger percentage of individuals are considered overweight or obese in developed countries, as developing countries tend to have a much larger populations, the absolute number of individuals who are overweight or obese in developing countries is much larger [2]. Developing nations are also subject to the same influences that contribute to the epidemic of overweight and obesity such as an increase in caloric intake, sedentary lifestyles and an aging population. In addition, as developing nations tend to have less funding for social programs and health care, this trend is expected to continue with drastic health consequences expected in the future [2-4].

When compared to the world population, Canadians do not fair much better. In 2004, 23% of adult Canadians (5.5 million people) over the age of 18 were obese with 36% (8.6 million people) overweight [5]. This is much higher than the data from the 1978 Canadian Health Survey which reported a Canadian obesity rate of 13.8% [6]. When separated by province, with a few exceptions, obesity rates did not differ much. In Canada, Newfoundland and Labrador, has one of the highest rates of obesity [6]. In 2005 the national body mass index (BMI) average was 22.9 but in Newfoundland it was 33.3.

~~This trend was also seen in Newfoundland women who also surpassed the national average with a BMI of 34.5 [6-7].~~

Health consequences related to obesity range from serious chronic conditions that reduce overall quality of life including diabetes, heart disease and hypertension to increased risk of premature death. [8]. Data from the 2004 Canadian Community Health Survey revealed that fewer than 10% of men and women with a normal ranged BMI reported having high blood pressure [7]. This is in contrast to 15% of overweight individuals surveyed and 20 % of obese individuals. This pattern was present in both men and women. In addition, the proportion of individuals who reported having type 2 diabetes (DM2) was almost triple that for obese individuals when compared to normal BMI individuals [7]. Further, overweight and obesity has been associated with an increase in primary care visits [9]. As the obesity problem increases, it is expected that the strain on the already over burdened health care system in Canada will increase as well. Finally, it has been determined that obesity produces an increased risk of premature death as recent research states that current life expectancy in Americans is now estimated for the first time to be shorter than parental generations due to the effects of obesity [10]. Even though Canadian obesity rates are lower than those of Americans, this fact could soon be true for Canadians.

1.2: Defining obesity.

Obesity is defined as an excessively high amount of body fat or adipose tissue in relation to lean body mass [11]. Currently there are various methods employed to measure obesity. The most commonly used measure is BMI. BMI is a measure of the

~~degree of overweight, in relation to the individual's height.~~ The formula used is $BMI = \text{weight}(\text{Kg}) / \text{height}^2(\text{m})$. It is used as a standard for assessing obesity due to its high correlation with weight, and simplicity of computation [3]. The WHO classifies normal weight as a BMI of 18.5-24.9 Kg/m^2 , overweight as 25.0-29.9 Kg/m^2 , and obese as a $BMI > 30.0 \text{ Kg/m}^2$ [3]. Many countries including Canada have adopted the WHO's BMI criteria for defining obesity.

As easy as BMI is to use, it is not without fault as it fails to account for a number of adiposity related-factors including age, gender, and ethnicity. BMI may inaccurately classify certain individuals as it is not a direct measure of adiposity or fat distribution. For example, body builders and other athletes with a high percentage of muscle mass and low body fat can be classified as obese by their BMI even though their body fat percentage (%BF) is very low [12]. Thus, a direct measure of %BF can be thought of as a more accurate indicator of a person's obesity status [13]. Reference methods such as dual energy x-ray absorptiometry (DXA), air-displacement plethysmography, and underwater weighing provide a more accurate indication of %BF which is directly linked to obesity and its associated disease risk [14-17].

The use of BMI for the classification of adiposity status and disease risk is based on epidemiological associations of BMI with morbidity and mortality [3, 18]. Despite this, numerous studies have produced evidence that BMI has limited ability to accurately predict body composition as evidenced by sizeable differences between BMI estimated body fat and densitometrically determined body fat [13, 19-21]. Furthermore, the relationship between BMI and %BF has been shown to vary with age, sex and ethnicity [22-24]. It is therefore essential to identify how well BMI criteria match with more

accurate reference methods based on %BF (%BF) and to what extent major factors such as age, gender and adiposity distort the accuracy of BMI. At the present time, there is little systematic data available in Canada regarding the accuracy of BMI compared to a standard reference method such as DXA, at the population level.

DXA is a precise, accurate, non-invasive, safe, and convenient technique for measuring %BF. DXA employs an X-ray tube to produce two X-ray beams; one high energy and one low energy beam. The different attenuation of these beams by the subject is measured by specific detectors and used to determine body composition [25]. The attenuation of X-rays by a subject depends on the subjects mass and body composition [26]. Body composition is then computed by the manufacturers software. Body composition data is delivered as bone mineral mass, fat mass and lean body mass and this data can be divided into various body compartments for further analysis.

DXA offers many advantages over other anthropometric measuring tools. DXA is very easy to perform with very little training or expertise needed. In order to operate the machine, one must only become familiar with the technique of positioning a subject in the proper way for a scan, how to input the relevant data needed about the subject into the software interface and how to initiate the scan from the software interface. A short lesson on calibration is also needed in order to be able to perform routine calibrations of the detecting system. Once the scan is complete, all data generated is automatically presented in raw and graphical data form (**Figure 1.1**).

DXA is also easy to perform on most subjects. Due to the design of the machine and the length of the scan, most subjects are able to tolerate the scan. Subjects are not enclosed and only have to lie fairly still on their backs while being scanned. The scan

~~can take anywhere from 5-20 minutes depending on the patients height and weight and~~
which scan is ordered. DXA is non-invasive so no special preparations are needed before the scan in terms of medications, anesthesia or contrast media. In addition, DXA is not dependent on other measurements such as height and weight which other measuring tools such as BMI and BIA are reliant on. The radiation dose for DXA is very low and ranges from 5-7 μ SV making its use widely applicable[26]. This dose is approximately 1/10th the dose of a standard chest X-Ray and less than a normal day exposure to natural radiation.

Although DXA offers many benefits, there are deterrents to its use in mainstream medicine. The most prominent deterrent is cost. However, with new industry competition, more companies are producing DXA machines reducing the costs associated with this technology. Although the radiation dose of DXA is low, there is still a concern with radiation exposure, especially in younger populations. In addition, measurement accuracy in some patient populations is a problem. DXA calculations assume that the hydration of fat-free tissue is constant at 73%. Hydration can range in various patients and if a subject contains more than the average amount of water, the software may over estimate the actual fat content [25]. As well, all DXA measurements assume that the amount of fat that lies over bone is similar and that of the adjacent soft tissue. This is not always true, for example, there can be a non-uniformity of adipose tissue distribution in the abdomen which can lead to unpredictable errors in measurement of spine bone mass density [27]. Lastly, for some patients, especially those with large body widths, DXA may over-estimate fat and bone mineral content at depths of greater than 20-25 cm[26]. Recent software changes have been developed to overcome some of these problems that are caused by tissue thickness [25-26, 28-29].

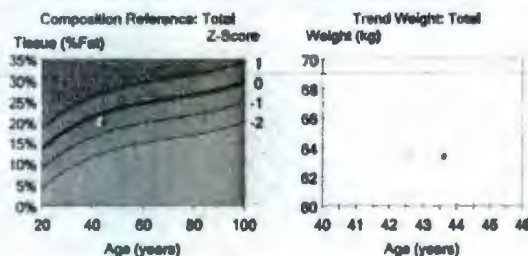
**Memorial University of Newfoundland, Discipline of
Faculty of Medicine, Room # HST-2B20
300 Prince Phillip Dr. St. John's, NL, A1B 3X6**

Patient's		Patient ID:	TEST
Birth Date:	10/05/1960 43.6 years	Physician:	DR NOAH, BODY
Height / Weight:	164.0 cm 63.5 kg	Measured:	06/01/2004 2:00:41 PM (6.70)
Sex / Ethnicity:	Male White	Analyzed:	06/01/2004 2:00:43 PM (6.70)

Total Body Tissue Quantitation



COMMENTS:



Region	Tissue (%Fat)	Z-Score	T.Mass (kg)	Fat (g)	Lean (g)	BMC (g)
Arms	14.0	-	-	961	5,895	362
Legs	13.7	-	-	2,505	15,835	953
Trunk	26.8	-	-	9,038	24,723	790
Total	20.5	-0.3	66.1	13,031	50,541	2,553

Trend: Total						
Measured Date	Age (years)	Tissue (%Fat)	Z-Score	T.Mass (kg)	Fat (g)	Lean (g)
06/01/2004	43.5	20.5	-0.3	66.1	13,031	50,541
15/01/2003	42.5	19.8	-	65.3	12,443	50,334

Image not for diagnosis

Printed: 14/01/2004 2:25:53 PM (6.70) 76.0 15:153.85:31.2 0.00:1.00
4.80x13.00 11.8%fat=26.5%
0.00x0.00 0.00x0.00
Filename: 0004_1020040104
Scan Mode: Standard

2 -USA, Total Body Reference Population
3 Matched for Age

GE Medical Systems
LUNAR

Prodigy
DF-14500

Figure 1.1: Example of dual-energy X-ray absorptiometry (DXA) scan output from a Lunar Prodigy (GE Medical Systems, Madison, WI, USA) DXA scanner.

~~Due to the ability of DXA to separate and accurately measure body composition,~~
 new classification criteria can be applied to determine obesity status. Bray produced obesity classification based on %BF which we used to classify our subjects. Brays classification allows for differences found between ethnic groups, genders and between age groups. Our subjects consisted of white males and females. Bray classified obesity for white females aged 20-39 years as $\geq 39\%$ body fat, for those aged 40-59 years as $\geq 41\%$, and for 60-79 year olds, it is $\geq 43\%$. Obesity for white males aged 20-39 years is classified as $\geq 26\%$ body fat, for those aged 40-59 years as $\geq 29\%$, and for 60-79 year olds, it is $\geq 31\%$ body fat (**Table 1.1**) [30].

Table 1.1. Percentage body fat (%BF) cut-off points for women and men¹.

Age (yrs)	Women (%BF)				Men (%BF)			
	Underweight	Normal Weight	Overweight	Obese	Underweight	Normal Weight	Overweight	Obese
20-39	<21	21-32	33-38	39+	<8	8-20	21-25	26+
40-59	<23	23-34	35-40	41+	<11	11-22	23-28	29+
60-79	<25	25-37	38-42	43+	<13	13-24	25-30	31+

¹Adapted from Bray 2003

1.3: Current evidence for the etiology of obesity.

A number of theories have evolved that attempt to explain the cause and or origin of the obesity epidemic. The focus of these different theories include explanations of evolutionary pressure, fetal programming, ethnic population size increases, a sedentary lifestyle or a combination of any or all of these ideas. These theories are reviewed in detail elsewhere [31-35]. Regardless of which theory one adopts, obesity is still considered a disorder of energy balance which results from either an excess of energy intake or a lack of energy expenditure. When energy intake exceeds energy output the excess is stored as triglyceride in adipose tissue. The World Health Organization attributes the increased prevalence of obesity to the increased consumption of more energy-dense, nutrient-poor foods containing high levels of sugar and saturated fats in combination with decreased physical activity [1]. However, not all of us who are exposed to excess calories or who have poor exercise habits become obese. Thus, other factors must influence body composition in addition to energy balance.

A link between obesity and genetics has been suspected for over 30 years (Feinleib *et al.*, 1977). Although environmental factors play a large part in the development of obesity, the evidence is clear that there is a large underlying genetic component and that heritability may play an important part in determining a child's risk of developing obesity. It is now well agreed upon that parental obesity is a strong risk factor for childhood and adolescent obesity and that the degree of parental obesity also influences this risk. This risk is further elevated if both parents are obese [36-37]. Although one might be led to believe that this has just as much to do with genetics as with environment and child rearing abilities, genetic studies have concluded that the predictive value of parental BMI on childhood obesity is mostly due to genetic rather

than environmental factors [38]. Some of the most compelling proof came from early twin studies by Stunkard *et al.* who estimated a heritability of 0.78 for weight [39]. Wardle has recently revealed similar heritability in children determining that genetics does play a significant role in development of obesity [40]. Wardle determined that even though today's environment has made children fatter than children 20 years ago, the main explanation for variations in BMI at the population level is genetic differences between individual children.

In addition to familial inheritance patterns, obesity patterns can also be seen between different races (Katzmarzyk *et al.*, 2000). Studies on body composition patterns show that black children have less fat than white children suggesting they have different disease risk stratifications [41-43]. In addition, Carroll *et al.* suggest race determined differences in visceral fat measurements. They found that middle aged African American men and women had lower visceral adipose tissue despite similar BMI and waist circumference measurements compared to the white and Hispanic groups studied. The altered relationship between anthropometric measures and visceral adipose tissue between races suggests that race and ethnicity should be considered when making determinations on obesity disease risk in different populations [44]. Moreover, Rush *et al.* found that the relationship between percentage body fat and BMI varied with ethnicity. Their study included 721 subjects from 2 countries and 5 ethnicities. The results suggested that a universal BMI may not be appropriate when comparing obesity prevalence among different ethnic groups. In other words, BMI does not provide a consistent reflection of adiposity and fat distribution across ethnic groups [45].

Although there is much compelling evidence for a genetic cause of obesity, until recently, no one gene has been identified as a causative agent for the majority of obesity seen in our population. What has been found is that most obesity genes appear to provide small contributions or cumulative contributions to human obesity phenotypes. These genes are thought to predispose an individual to obesity rather than to be the dominant cause. Put simply, the genes load the gun but environmental factors pull the trigger [46]. However, recent evidence from two independent studies using genome wide association scans has revealed that variations in the fat mass and obesity associated gene (*FTO*) are associated with common forms of human obesity [47-48]. This is thought to be the first evidence of a single gene directly contributing to common forms of obesity [49]. *FTO* is thought to be responsible for 1% of total variance of BMI [50]. Thus a more generally accepted theory is that a combination of diet, lifestyle and inheritance contributes to the development of complex diseases such as obesity.

1.4: Association studies and the identification of obesity genes

There are various ways to study genetic determinants of disease however, association studies are a commonly used tool. In their most basic form, association studies compare the frequency of alleles or genotypes of a particular variant between disease cases and controls (Hirschhorn and Daly, 2005). So far, association studies have been successful in identifying many genetic risk factors for common diseases such as apolipoprotein E (APOE) for the age of onset and risk of Alzheimer's disease (Strittmatter and Roses, 1996), peroxisome proliferator-activated receptor-gamma 2

(PPAR γ 2) for DM2 (Deeb *et al.*, 1998), and recently the association of FTO gene and obesity (Frayling 2007, Scuteri 2007).

Risch and Merikangas argued that the association study offers a more powerful means of identifying genetic variants that influence a person's susceptibility to complex diseases over traditional methods such as linkage analysis (Risch and Merikangas, 1996). Adding to this argument, Burton *et al.* state that it is more likely the genetic component of obesity has a cumulative effect and this effect likely increases susceptibility to obesity rather than be the direct cause [51]. It is the increase in susceptibility that variations in candidate genes may cause that is the focus of obesity association studies. To address these small cumulative effects in candidate genes, association studies have begun to incorporate larger and larger sample sizes such as the recent work by Frayling *et al.* and the discovery of the FTO gene association with obesity. This study included over 38000 individuals [47].

Although seemingly successful, the association study has been questioned due to non-replication of results as well as limitations in the ability to include all possible causative genes and polymorphisms[52]. Explanations for lack of reproducibility usually include small sample size, random error, poorly matched control groups, over-interpretation of data, and positive publication bias [53-54]. However, a good study design can overcome all of these problems [55]. In addition, problems with population substructure have raised concerns for case control associations [53]. Allelic frequency variation between cases and controls may reflect differences in their ancestry rather than a relationship to a phenotype of interest [51, 53]. This problem can be reduced by

sampling a single ethnic group or race to produce a more genetically homogeneous sample [53-54].

Association studies are classified as either genome-wide or candidate-gene association studies [56]. Genome-wide association studies are a hypothesis free approach requiring the screening of the whole genome with the aim of identifying new, unanticipated genetic variants associated with a given disease or trait [49]. This approach is a powerful method for identifying genes involved in common diseases, such as obesity, since no assumptions are made about their genomic location or their function. As it does not rely on familial relatedness, genome wide association studies can use large cohorts of cases and controls than typical family based studies. The two major obstacles facing the genome-wide approach are the enormous associated cost and the issue of type one errors (false positives) when performing multiple tests. However, recent advances in technology and increased market competition have significantly reduced the cost of doing genome wide association studies.

New Candidate-gene studies rely on hypotheses generation and identification of candidate genes that have a role in the etiology of the disease in question [52]. This approach entails genotyping genetic variants, such as single nucleotide polymorphisms (SNPs), in or near causative genes in a population and determining whether there is a statistical relationship between those variants and the disease phenotype.

1.5: Adipose Tissue – Adipocyte metabolism and the PAT protein family

Many genes have been identified by the Human Obesity Gene Map as possible candidate genes in the pathogenesis of obesity [57]. When choosing a candidate gene, it

~~is best to start with the basics. One of the most basic aspects of the pathogenesis of~~
obesity is the way fat is stored and released in the body. Adipose tissue is the largest energy reservoir in the body [58]. It is found in mammals in two forms: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT makes up the majority of adipose tissue in the body [59]. However, it has recently been reported that the adult human body contains much more BAT than once thought and the role of BAT in obesity is being reanalyzed [60]. The focus of this study is WAT and the way its cells (adipocytes) are metabolized.

Adipocytes function primarily to synthesize and store lipids when nutrients are abundant, and release fatty acids when nutrients are needed [61]. When caloric intake consistently exceeds expenditure, excessive adipose tissue will accumulate. Under conditions of energy abundance, the structure of the adipocyte changes and it will swell (to up to six times the original size) and multiply in numbers from 40 billion (average adult) to as many as 100 billion [62]. It is also known that excessive adipose tissue increases one's risk for numerous medical conditions such as coronary artery disease, hypertension, dyslipidemias, DM2, and cancer [63].

Adipose tissue is not just an energy storage site but it is also considered a heterogeneous organ. It has been reported that in addition to adipocytes, human adipose tissue contains a population of non-characterized cells that are able to undergo adipogenic, osteogenic, chondrogenic or miogenic differentiation *in vitro* [64]. These cells are collectively called the stromal-vascular fraction, which also include pre-adipocytes, endothelial cells, and macrophages [65]. WAT is now recognized as a highly dynamic organ, with involvement in metabolic regulation and physiological homeostasis

~~[64]. It was the discovery of the cytokine-like hormone leptin which is secreted from~~
adipocytes, that helped shape the theories of adipose tissue as an endocrine organ [66-67]
. Since then, numerous other adipocytokines have been discovered. The classification of
these adipocytokines by functional role include appetite and energy balance, immunity,
insulin sensitivity, angiogenesis, inflammation and acute-phase response, blood pressure,
lipid metabolism, and hemostasis [66, 68]. The main focus of our research was to
investigate how adipocytes store and release excess food energy and if the genes involved
influenced these actions.

To determined how adipocytes store and release energy, the internal structures of
the fat cell need to be looked at more closely. Adipocytes contain specialized organelles
known as lipid storage droplets, which regulate the storage and release of intracellular fat
stores. Lipid storage droplets (LSD) are intracellular organelles found in most cell types.
These organelles serve mainly as triacylglyceride (TAG) reserves for times of increased
energy need and LSDs in adipocytes contain the largest reserves of TAG in the body
[69]. Not only is storing fat in cells advantageous in times of energy need, but the stored
lipid can also be used as substrate for synthesis of other important cellular molecules,
such as membrane phospholipids. In addition, the products of TAG hydrolysis,
(diacylglycerol (DAG) and free fatty acids), may influence cell signaling either directly
or via subsequent metabolism, (ex fatty acyl coenzyme A). Free fatty acids can also
influence gene expression by acting as ligands for nuclear receptors, such as the
peroxisome-proliferator activated receptor (PPAR) family. Finally, excess intracellular
free fatty acids can disrupt phospholipid bilayer membrane integrity, alter lipid signaling
pathways, and induce apoptosis [70]. Thus, LSDs provide cells a way of regulating the

~~availability of substrates for energy utilization and for lipid signaling molecules as well~~
as for potentially toxic metabolites.

Lipolysis is the hydrolysis of fatty acyl esters and is the means to which stored lipids are mobilized from LSDs [71]. Lipolysis of TAG is needed for energy release and is controlled by the activation of Adipose Triglyceride Lipase (ATGL) and Hormone Sensitive Lipase (HSL) by protein kinase A (PKA) [72-74]. In addition, intracellular lipolysis is tightly regulated by proteins which coat the LSD. These proteins are collectively known as the PAT family of proteins, a term first coined by Lu *et al.* [75]. This family is composed of five proteins (perilipin, adipophilin, tip-47, s3-12 and OXPAT) [71]. These proteins all share sequence similarity and the ability to bind lipid droplets. These proteins all differ from one another in size, tissue expression, affinity for LSDs and transcriptional regulation suggesting that each PAT protein has distinct cellular functions [69, 71]. However, each protein contains a highly conserved sequence of amino acids called the PAT domain and this domain is the major site of interaction with PKA and HSL [71]. The PAT family is evolutionarily ancient and is found in many animal species. The conservation of the PAT family throughout evolution helps place emphasis on the importance of its role in the regulation of intracellular lipid stores. We chose to study genetic variations within two proteins from the PAT family, namely perilipin and adipophilin (ADFP) and to determine if there was significant association between these variations and obesity phenotypes.

1.6: ~~Candidate gene association approach~~ – The perilipin gene

Lipolysis in adipocytes is facilitated by three lipases ATGL, HSL and monoacylglycerol lipase (MGL). Lipolysis is a tightly regulated process and the current understanding is that the process of lipid mobilization from LSDs occurs in a multiple step process starting with catecholamine signaling via a β -adrenergic receptor and a G-protein signaling cascade that elevates intracellular levels of cAMP [71, 76]. Increased cAMP levels in turn activate PKA which phosphorylates LSD coating proteins and HSL. HSL then translocates from the cytoplasm to the surface of the LSD. It was originally thought that the activation and translocation of HSL was the initiation step to lipolysis but recent evidence has revealed that a second lipase, ATGL, is the principle lipase in TAG lipolysis [77-79]. ATGL is needed for the first step in TAG hydrolysis whereas HSL then acts on DAG followed finally by MGL which is required for the final hydrolysis of the 2-monoacylglycerol produced by HSL activation [80].

Perilipin (PLIN), a hormone regulated phosphoprotein, has been shown to coat the outer surface of LSDs [71, 81-82]. PLIN protein is found only in adipocytes and steroidogenic cells [83]. In adipocytes, the most abundant LSD coating protein is PLIN. PLIN is coded for by the single *PLIN* locus located on chromosome 15q26, a region previously linked to increased susceptibility to obesity and DM2 [71]. *PLIN* codes for a three isoforms of PLIN (perilipin A, B, and C) that arise from translation of alternatively spliced mRNA [75, 83]. The protein named Perilipin A is the largest isoform (522 amino acids) and the most abundant form found in adipocytes [83-84]. All three PLIN isoforms share a common N-terminal region but differ in their C-terminal tails [83]. Perilipin A is the most studied isoform of the perilipin family.

Expression of *PLIN* is regulated primarily by peroxisome proliferator activated receptor gamma (PPAR γ) and is closely linked to the storage of neutral lipids in adipocytes [85]. All three *PLIN* isoforms are rapidly degraded when not bound to lipid droplets [86]. Thus, the mass of the stored neutral lipid acts as a means of post-translational control of perilipin protein levels in adipocytes.

Perilipin has 2 roles in triglyceride storage in adipocytes. The first is a protective role, where perilipin coats the surface of the LSD thereby stabilizing the neutral lipid stored in the LSD. The second is a mobilization role, where perilipin works in concert with lipases to release free fatty acids from the LSD. Current theories suggest that by coating LSD, perilipin protects them from breakdown until lipolysis is activated [81, 87-88]. Brasaemle showed that cultured fibroblasts lacking endogenous perilipin increased their TAG content by slowing the rate of TAG turnover after addition of ectopic perilipin A [88]. *In vitro* studies in *PLIN* knockout mice have produced lean mice that were non-responsive to diet [81]. In addition, perilipin null mice show an elevation of basal lipolysis by a factor of 10 and a 75% decrease in adipose tissue when compared to wild type animals. Perilipin knockout mice also have a blunted response to catecholamine induced lipolysis in their adipocytes [89-90]. New research from Miyoshi *et al.* has shown that *PLIN* overexpression in mice protects against diet induced obesity [91]. They noted that *PLIN* overexpression in adipose tissue protected against diet-induced adipocyte hypertrophy, obesity and glucose intolerance in transgenic mice suggesting a novel role for *PLIN* in adipose tissue metabolism and regulation of obesity.

Catecholamine driven activation of lipolysis initiates the PPAR γ signaling pathway resulting in the activation of PKA. Perilipin A has six sites for phosphorylation

by PKA [92]. Once phosphorylated by PKA perilipin A no longer acts to protect lipid stores but rather assists in their mobilization. Phosphorylated perilipin A also acts as a recruiter for HSL resulting in its translocation from the cytosol to the surface of the LSD [93]. Sztalryd's lab showed that HSL failed to translocate from the cytosol to the surface of LSDs in cells lacking perilipin. They further confirmed the role of perilipin in HSL translocation by using CHO fibroblast cells transfected with different forms of perilipin A and Green Fluorescent Protein labeled HSL (GFP-HSL). Cells were transfected with wt perilipin A, perilipin A with the final 3 PKA phosphorylation sites mutated and with perilipin null mutations. The results showed that GFP-HSL translocation only occurred in CHO cells expressing fully phosphorylatable perilipin A coated LSDs.

Variations in the *PLIN* gene have also been associated with numerous cardiometabolic risk factor phenotypes. Jang et al. found that fasting plasma free fatty acid changes following a modest weight loss in overweight-obese subjects were influenced by the genetic variability at the *PLIN* locus [94]. Moreover, Corella found that certain variations in the *PLIN* gene were more resistant to weight loss than others, suggesting that certain polymorphisms may predict outcome of weight loss strategies based on low-calorie diets [95]. This was found to be true for different ethnicities as well and suggested a possible gender bias towards women [96-98]. In addition, Perez-Martinez *et al.* found that the presence of the minor allele in certain *PLIN* variations was significantly associated with lower postprandial TAG levels suggesting a decreased atherogenic risk in these individuals over the major allelic variation [99].

PLIN variations have been implicated as a factor in insulin resistance as well. Corella found evidence of an interaction between dietary fat (specifically saturated fat)

~~intake, polymorphisms at the perilipin locus and insulin resistance [100].~~ They determined that a greater intake of energy from saturated fats was associated with increased insulin resistance amongst individuals who were homozygous for the rare alleles but not individuals who carried the common allele. These interactions were observed only in women providing more evidence of a possible gender bias seen with *PLIN* variations. In addition to different effects on obesity associated diseases, *PLIN* has also been implicated as having a role in bone density [101], and in immunohistochemical staining for the presence of sebaceous gland carcinoma [102].

Current evidence suggests that genetic polymorphisms in the *PLIN* gene could play a critical role in the etiology of human obesity. Taking this evidence into account, our study chose to investigate the effect of three Single Nucleotide Polymorphisms (SNPs) in the *PLIN* gene on obesity phenotypes in a large, healthy, homogeneous population from the Canadian province of Newfoundland and Labrador.

1.7: Candidate-gene association approach – The adipophilin gene

Adipose differentiation related protein (ADRP) is a 50 kDa protein first identified as an RNA transcript that was significantly induced during differentiation of cultured adipocytes in mice models [103-104]. Sequence similarity to perilipin led to the discovery that ADRP coats small lipid storage droplets [86]. A human analog was found and later renamed adipophilin or ADFP for short [105]. Unlike perilipin, whose expression is limited to adipocytes and steroidogenic cells [83], ADFP is expressed in a number of different tissue types including early differentiating adipocytes [105-107].

~~Adipophilin~~ expression was found to be induced by long-chain free fatty acids (FFA) in different cell lines [108-109]. The result after FFA stimulation was an upregulation of *ADFP* mRNA within the cell. This increase in *ADFP* mRNA occurs in conditions in which FFA flux is high, such as fasting *in vivo* or FFA incubation *in vitro*. However, as adipocyte differentiation progresses and *ADFP* mRNA levels increase, *ADFP* protein levels decrease [104, 107]. This is in contrast to perilipin, for which both its mRNA and protein levels increase as adipocytes mature [107]. During adipocyte differentiation perilipin protein replaces the initial *ADFP* protein coating the LSDs until there is no *ADFP* seen associated with LSDs [86]. This discovery is reinforced by the fact that *ADFP* protein is known to associate with smaller neutral LSDs located within most tissues, but rarely in adipose cells that express perilipin [107]. Furthermore, mature adipocytes lacking perilipin were found to have significantly smaller LSDs which were coated by *ADFP*. When exogenous perilipin is added to these cells, the *ADFP* is replaced by the perilipin suggesting that perilipin out competes *ADFP* for the surface of the LSD [89, 110-111]. This also suggests that *ADFP* is ubiquitously expressed in all cell lines [107].

ADFP is associated with TAG droplets in non-adipocytes [107]. However, *ADFP* does not offer the same PKA regulation of lipolysis as does perilipin [89] and in fact *ADFP* is not phosphorylated by PKA [89, 93]. Similar to perilipin, any non-LSD bound *ADFP* is quickly degraded [112-113]. Xu *et al.*, found that protection of *ADFP* from degradation with the proteasome inhibitor MG-132 resulted in a substantial increase in lipid content (170%) compared with control cells. This finding suggested that *ADFP*, like perilipin, protects neutral lipid stores from degradation by lipases [112, 114].

However, cells lacking functional perilipin have significantly lower TAG stores than wt cells due to increased basal lipolysis levels. This suggests that ADFP does not offer the same level of protection as does perilipin from basal lipolysis [89, 110-111].

The exact role of ADFP in the cell has not yet been determined experimentally. Although, it does appear that ADFP protects neutral lipid stores from degradation within the cell, it is thought the role of ADFP is more focused towards the accumulation of TAG stores in LSDs. For example, over-expression of a green fluorescent protein labeled ADRP in Swiss-3T3 cells resulted in increased TAG content, even when the cells were cultured in non-lipid containing serum [115]. Also, the presence of ADRP precedes that of perilipin during the assembly of LSDs in 3T3 pre-adipocytes [86, 115]. Listenberger *et al.* found that over expression of ADFP in human embryonic kidney cells resulted in increased TAG levels and reduced TAG turnover. They also found that increased ADFP production reduced the association of ATGL with LSDs suggesting a further mechanism of how ADFP protects LSDs from releasing TAG [116].

ADFP also has been shown to play a major role in foam cell formation and has been described as a possible proatherosclerogenic protein. Various publications support this statement. It has been shown that ADFP expression increases in response to different forms of lipid loading in primary human monocytes [117] and in several macrophage/monocytic cell lines [118-120]. Beuchler *et al.* showed that ADFP could be induced in macrophage foam cells and that expression increased in response to different forms of lipid loading in primary human monocytes. They treated human monocytes with enzymatically modified low-density lipoprotein and found they could induce foam cell formation in monocytes and upregulate ADFP mRNA and protein within 2 h of

incubation. The rapid induction of ADFP was accompanied by a significant increase of free fatty acid. Beuchler *et al.* hypothesized that ADFP facilitates the uptake of free fatty acids, and the increase in intracellular free fatty acids was related to the early upregulation of ADFP expression in serum monocytes [117].

Larigauderie *et al.* showed that ADFP over expression increased lipid accumulation in THP-1 macrophages upon incubation with acetylated LDL, whereas depletion of ADFP using small interfering RNA (siRNA) reduced lipid accumulation [119]. Furthermore, their analysis of carotid endarterectomy specimens showed that ADFP expression was 3.5-fold higher in atherosclerotic plaques than in healthy areas of the same artery [119]. Analysis of human carotid endarterectomy and coronary artery specimens by Wang *et al.* also demonstrated high ADFP expression and found that it was localized mostly in a subset of lipid-rich macrophages [121]. Adding to this evidence, Chen *et al.* found that over expression of adipophilin in macrophages augments expression and secretion of proinflammatory cytokines in macrophages [122]. Lipid accumulation and ADFP expression in macrophages positively correlated with one another regardless of whether lipid accumulation was being driven by lipid loading or whether ADFP levels are being manipulated by over expression or RNA interference and in turn this appeared to lead to proinflammatory changes seen in atherosclerotic plaques.

ADFP has also been implicated as an early marker for the development of DM2. Varela recently found that ADFP knockout mice had decreased levels of TAG and diacylglycerol in their livers, but fatty acids, long-chain fatty acyl CoAs, ceramides, and cholesterol levels were unchanged [123]. They also determined that insulin action was enhanced in the livers of these mice, whereas insulin action in muscle and adipose tissue

~~was not affected. Other interesting research in this area has shown that interventions~~
associated with improved insulin sensitivity in humans, specifically weight loss in obese, non-diabetic subjects or treatment of DM2 subjects with insulin sensitizers, are associated with increased skeletal muscle ADFP protein levels. Phillips *et al.* treated nine DM2 subjects with known insulin sensitizers (troglitazone or metformin) in combination with a sulfonylurea (glyburide) for up to 12 weeks. Levels of muscle ADFP in muscle increased by 73% after insulin sensitizer treatment in the diabetic cohort and the averaged ADFP expression increased 192% over baseline in the weight loss cohort [124]. An improvement that Phillips correlated to increased levels of ADFP that acted by improving the efficiency of lipid storage and lipolytic control. In contrast, Minnard showed that rosiglitazone treatment decreased muscle ADFP content in diabetes patients [125]. Obviously work is still needed in this field.

To date there are no published reports of associations between genetic variations in the ADFP gene and obesity in humans. Taking into account the evidence that ADFP is involved in the production and homeostasis of TAG stores in different cell lines, our study chose to investigate the effect of two single nucleotide polymorphisms (SNPs) in the *ADFP* gene on obesity phenotypes in a large, healthy, homogeneous population from the Canadian province of Newfoundland and Labrador.

1.8: The association of abnormal serum lipids with increased serum calcium levels

An abnormal lipid profile, known as dyslipidemia, is defined as an abnormal reading of one or more serum lipid parameters. These parameters include elevated plasma cholesterol, LDL-cholesterol (LDL-c) and TAGs or a low HDL-cholesterol (HDL-c) level. Primary dyslipidemia refers to the overproduction and/or decreased clearance of

plasma lipoproteins [126-128]. For example, primary dyslipidemias can be found in several monogenic disorders that lead to different types of dyslipidemias such as familial hypercholesterolemia [126-128]. In many cases the etiology is polygenic. Secondary dyslipidemias can be defined as mixed elevations of cholesterol and TAG as a response to or resulting from an underlying medical condition such as DM2 or hypothyroidism. They can also be the result of effects from drug treatments with drugs such as thiazide diuretics or from chronic alcohol abuse [129]. These conditions include, but are not limited to, DM2, obesity, alcohol overuse, chronic renal insufficiency and/or failure, hypothyroidism, liver diseases, rheumatoid arthritis, and pregnancy. Secondary dyslipidemias can also be caused by the use of drugs such as thiazides, β -blockers, retinoids and sex hormones [130-135]. Cholesterol and other serum lipids can come from the diet as well as be produced by endogenous synthesis and this synthesis occurs mostly from ingested fat and sugar.

A cluster of diseases and syndromes including hypertension, insulin resistance, DM2 and atherosclerosis often coexist or overlap in their clinical symptoms [136-140]. Lind *et al.* found that measurements of blood pressure, serum glucose and serum cholesterol were all positively associated with each other [141]. However, the specific biochemical pathways that are dysregulated and the molecular mechanisms that lead to the dysfunction in dyslipidemias, except for those caused by clear genetic and secondary conditions, remain unclear. Calcium is a versatile intracellular messenger that is involved throughout the life cycle of an organism to control diverse biological processes [142]. It has been suggested that diabetes and cardiovascular disease are linked by a common defect of divalent cation metabolism, including calcium [143].

~~Insulin resistance, a state in which increased concentrations of insulin are required to~~ produce a given biological response, has been the focus of attention for a common link between several clinical disorders with dyslipidemia as one group of the clinical manifestation [144-146]. A previous study published by our lab has shown that altered serum calcium homeostasis is at least partially responsible for insulin resistance in the general population [147]. Data from a Swedish group supported our findings [148]. Together, this research helps strengthens the theory that calcium might be the central link between dyslipidemia and insulin resistance in hypertension, DM and atherosclerosis. Some preliminary association at the population level has been reported in previous studies, including the Framingham Heart Study [149], where relationships between calcium and general biological markers [150], and metabolic syndrome [141] were found. However, the general association was not further discussed and left many questions unanswered because of the complicated nature of lipid abnormality and serum calcium variation. Variations of lipids and serum calcium can be potentially influenced by many factors including age, gender, obesity status, medication use, physical activity level and other diseases such as DM [130-135].

To further investigate the relationship between serum lipids and serum calcium we designed a study that would control for major confounding factors such as medications, disease status, menopause, parathyroid hormone (PTH) and 25-OH-vitamin D status in addition to the common confounding factors of age and gender.

2a: Comparison of the classification of obesity by BMI versus dual-energy x-ray absorptiometry.**2a.1: Subjects**

For this study, a total of 1691 adults were recruited from an ongoing large-scale nutritional genetics study of human complex diseases called the CODING (*Complex Diseases in the Newfoundland population: Environment and Genetics*) study [151]. Subjects were recruited from the province of Newfoundland and Labrador (NL), Canada, by means of poster distribution and person-to-person contact. Subjects who met the following criteria were eligible to participate in the study: 1) aged 19 or older; 2) at least third generation Newfoundlander; 3) healthy, without any serious metabolic, cardiovascular, or endocrine disease and (4) not pregnant at the time of the study. All subjects provided written consent and completed questionnaires pertaining to their demographic background, health status, and family history. The Research Ethics Board of the Faculty of Medicine, Memorial University of Newfoundland, approved the study prior to commencement.

2a.2: Study design

This study investigated differences between BMI-determined adiposity status and DXA to evaluate the accuracy of BMI. The objectives of our study were as follows: 1) Determine the accuracy of BMI classifications compared to %BF classifications measured by DXA; 2) Determine if discrepancies between BMI and DXA are gender-

and age specific; 3) Identify whether an individual's current adiposity status (i.e. overweight or obese) can affect the size of error in their BMI.

2a.2.1: Body composition measurements

Subjects were weighed, and their heights were measured, wearing standardized light gowns, without shoes, on a platform manual scale balance (Health o meter Inc., Bridgeview, IL, USA). Whole-body composition measurements, including %BF, trunk fat percentage (%TF), lower body (legs) fat percentage (%LF), lean body mass, and bone mineral densities were determined using dual-energy X-ray absorptiometry (DXA) by Lunar Prodigy (GE Medical Systems, Madison, WI, USA). Measurements were performed on subjects, following the removal of all accessories containing metal, while lying in a supine position. Software version 4.0 was used for analysis.

2a.3: Statistical analysis

Prior to data analysis, all subjects were classified according to adiposity status using both BMI and %BF criteria. Subjects were classified using BMI as underweight (UW; $< 18.5 \text{ kg m}^{-2}$), normal weight (NW; $18.5 - 24.9 \text{ kg m}^{-2}$), overweight (OW; $25.0 - 29.9 \text{ kg m}^{-2}$), or obese (OB; $> 30.0 \text{ kg m}^{-2}$) according to criteria of the World Health Organization [3]. Subjects were grouped according to %BF based on criteria recommended by Bray that is both age and gender specific (**Table 1.1**) [30]. Differences in physical characteristics between men and women were assessed using Student's t-test. Differences in body weight classification between BMI and DXA were analyzed on the following three levels:

1. ~~Discrepancy analyses between BMI and DXA within gender.~~

Men and women were separated into weight classifications according to BMI and %BF criteria. The number of subjects grouped into each weight category by both methods was calculated as a percentage of the total number of participants. Differences in percentages between BMI- and %BF-defined adiposity status were analyzed within gender using Chi-Square analyses.

2. Discrepancy analysis by age group.

BMI-defined weight classifications were compared to %BF criteria among different age groups to investigate the effect of age on BMI accuracy. Women were separated into four groups according to their age (20-29.9, 30-39.9, 40-49.9, 50+) and differences in percentages between BMI- and %BF-defined adiposity status were analyzed using Chi-Square analyses. Due to the small number of men in our cohort, similar analysis in males could not be performed as the number of subjects in each cell (four age groups by four weight groups) was too small for effective comparison.

3. Ranges of percent body fat based on BMI cutoffs.

In order to study the range of %BF found in each BMI category, subjects were grouped by BMI into adiposity groups and then %BF averages for each BMI group were calculated along with minimum and maximum values.

SPSS version 14.0 (SPSS Inc., Chicago, IL) was used for all analyses. Statistical analyses were two-sided and a p value < 0.05 was considered to be statistically significant.

2b: Association between serum lipids and serum calcium levels.

2b.1 Subjects

For this study, a total of 1907 adults were recruited from an ongoing large-scale nutritional genetics study of human complex diseases called the CODING (*Complex Diseases in the Newfoundland population: Environment and Genetics*) study [151]. Subjects were recruited from the province of Newfoundland and Labrador (NL), Canada, by means of poster distribution and person-to-person contact. Subjects who met the following criteria were eligible to participate in the study: 1) aged 19 or older; 2) at least third generation Newfoundlander; 3) healthy, without any serious metabolic, cardiovascular, or endocrine disease and (4) not pregnant at the time of the study. All subjects provided written consent and completed questionnaires pertaining to their demographic background, health status, and family history. BMI was calculated as body weight (kg) divided by height (m) squared. Subjects who self-reported having DM or who had fasting glucose levels ≥ 7.0 mmol/L were designated as having DM. The Research Ethics Board of the Faculty of Medicine, Memorial University of Newfoundland, approved the study prior to commencement.

2b.2: Biochemical measurements

Blood samples were obtained from all subjects after they had fasted for 12 h. Serum concentrations of total calcium, phosphorus, magnesium, triglycerides, total cholesterol, and HDL-c were measured. Additionally, glucose and albumin were measured using Synchron reagents performed on an LX20 clinical chemistry analyzer (Beckman Coulter, Fullerton, CA). Serum total calcium concentration was corrected by

serum albumin using the following formula: $\left[\text{calcium} + 0.1 \times \frac{(\text{10 albumin})}{6} \right]$ [147].

LDL-c was calculated by using the following formula: $\text{cholesterol} - \text{HDL}_c - \left(\frac{\text{triglycerides}}{2.2} \right)$. The LDL-c calculation is reliable in the absence of severe hyperlipidemia. Serum parathyroid hormone (PTH) and insulin were measured on an Immulite immunoassay analyzer using the IMMULITE 1000 Intact PTH kit (Diagnostic Products Corporation, Los Angeles, CA). 25-OH-vitamin D was measured using a 25-Hydroxyvitamin D 125 I RIA Kit (DiaSorin, Stillwater, MN). Homeostasis model assessment was used to estimate insulin resistance and β -cell function [152].

2b.3: Measurement of body composition

Subjects were weighed, and their heights were measured, wearing standardized light gowns, without shoes, on a platform manual scale balance (Health o meter Inc., Bridgeview, IL, USA). Whole-body composition measurements, including %BF, trunk fat percentage (%TF), lower body (legs) fat percentage (%LF), lean body mass, and bone mineral densities were determined using dual-energy X-ray absorptiometry (DXA) by Lunar Prodigy (GE Medical Systems, Madison, WI, USA). Measurements were performed on subjects, following the removal of all accessories containing metal, while lying in a supine position. ENCORE 2002 software version 6.70 was used for analysis.

2b.4: Statistical analysis

Data are presented as means \pm SD. The analyses consist of the following parts:

-
1. ~~Partial correlation analyses were performed between the levels of serum calcium~~
and the levels of total cholesterol, TAG, HDL-c and LDL-c within sex after
controlling for the confounding variables.
 2. Partial correlations were repeated according to medication use and menopausal
status (pre- and post-menopausal women), within group with or without
medication use, and within each group of pre- or post-menopausal women.
 3. In one-way ANOVA analyses, adjustments for the effects of covariates on serum
calcium, cholesterol, TAG, HDL-c and LDL-c were carried out using a linear
regression procedure. The covariates for serum calcium and lipids were age,
phosphorus, and magnesium; percent trunk fat was also included as a covariate in
the analyses for serum lipids. The results from one-way ANOVA were corrected
by the TUKEY method.
 4. To account for the influences of vitamin D and PTH, subjects from the
“medication-free” group were ranked according to serum calcium concentration;
100 women (top 50 and bottom 50) were selected, and the serum concentrations
of 25-OH vitamin D and PTH were measured. Total serum calcium levels were
adjusted for the concentrations of 25-OH vitamin D and PTH using a linear
regression model before analysis. The levels of total cholesterol, TAG, HDL-c
and LDL-c were then compared between the low and high calcium groups using a
student *t* test. All analyses were performed using the SPSS software for Windows,
version 15.0.

2b.5: Confounding factors

Of all the confounding factors considered in this study, the use of medications is probably the biggest factor that modifies serum lipids either primarily by treating dyslipidemia or secondarily through other medical processes such as hormone replacement therapy in women. In addition to serum calcium, evidence from both human and animal experiments show that magnesium may exert influence on lipid metabolism [153-156]. Obesity status is the most prominent risk factor contributing to metabolic syndrome and lipid abnormalities, especially central obesity [130, 157]. Trunk fat percentage was found to be more significantly associated with lipids in our primary analysis using Pearson correlation than other measures of body composition. Therefore, we chose trunk fat percentage as the covariate representing body composition.

2c: Genetic association study – Roles of Perilipin and Adipophilin on obesity phenotypes

2c.1: Subjects

For this study, a total of 1273 adults were recruited from an ongoing large-scale nutritional genetics study of human complex diseases called the CODING (*Complex Diseases in the Newfoundland population: Environment and Genetics*) study [151]. Subjects were recruited from the province of Newfoundland and Labrador (NL), Canada, by means of poster distribution and person-to-person contact. Subjects who met the following criteria were eligible to participate in the study: (1) aged 19 years or older, (2) at least third generation Newfoundlander, (3) healthy, without any serious metabolic, cardiovascular or endocrine diseases, and (4) not pregnant at the time of the study. All subjects provided written consent and completed questionnaires pertaining to their

demographic background, health status, and family history. The Research Ethics Board of the Faculty of Medicine, Memorial University of Newfoundland, approved the study prior to commencement.

2c.2: Study design

This study was a candidate-gene association study on a large NL based cohort. All measurements and blood samplings were performed in the morning following a 12-hour fast.

2c.2.1: Blood samples

For plasma isolation, blood was collected in evacuated tubes containing K₃EDTA as an anti-coagulant. Tubes were immediately centrifuged at 4 °C for 15 minutes at 1300g. Plasma was removed and stored at -80 °C until further analysis. For serum isolation, blood was collected in tubes containing a clot activator. Tubes were allowed to sit at room temperature for 20 minutes, followed by centrifugation at 25 °C for 10 minutes at 3500 rpm. Serum was removed and stored at -80 °C until further analysis.

2c.2.2: Body composition measurements

Subjects were weighed, and their heights were measured, wearing standardized light gowns, without shoes, on a platform manual scale balance (Health o meter Inc., Bridgeview, IL, USA). Whole-body composition measurements, including %BF, trunk fat percentage (%TF), lower body (legs) fat percentage (%LF), lean body mass, and bone mineral densities were determined using dual-energy X-ray absorptiometry (DXA) by

Lunar Prodigy (GE Medical Systems, Madison, WI, USA). Measurements were performed on subjects, following the removal of all accessories containing metal, while lying in a supine position. Software version 4.0 was used for analysis. Classification for lean and obese were as follows: obese for females aged 20-39 years was classified as $\geq 39\%$ body fat (lean as $< 33\%$) and aged 40-59 years was classified as $\geq 41\%$ (lean as $< 35\%$); obese for males aged 20-39 years was classified as $\geq 26\%$ body fat (lean as $< 21\%$) and aged 40-59 years was classified as $\geq 29\%$ (lean as $< 23\%$) (Table 1) [30].

2c.2.3: Biochemical measurements

Serum concentrations of glucose, total cholesterol, HDL-C, and TAGs were measured using Synchron reagents performed on an Lx20 (Beckman Coulter, Inc., CA, USA) by Dr. Edward Randell in the discipline of Laboratory Medicine. Serum insulin levels were measured on an Immulite immunoassay analyzer (DPC, CA, USA). LDL-C was determined using the following equation: $[\text{total cholesterol} - \text{HDL-C} - (\text{TAGs}/2.2)]$, and risk factor was determined using the equation: $(\text{total cholesterol}/\text{HDL-C})$.

2c.2.4: Genomic DNA extraction and genotyping

Genomic DNA was extracted from whole blood (5ml) using the Wizard® Genomic DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. This kit is designed for the isolation of DNA from white blood cells. The protocol can be summarized in four main steps. Firstly, red blood cells were lysed in the Cell Lysis Solution and removed. Secondly, white blood cells and their nuclei were lysed in the Nuclei Lysis Solution. Thirdly, cellular proteins were removed

by a salt precipitation step, leaving genomic DNA in solution. Finally, the genomic DNA was concentrated and desalted by an isopropanol precipitation.

Genotyping was performed using the TaqMan® validated SNP Genotyping Assays (Applied Biosystems, CA, USA) according to the manufacturer's protocol. Each assay required polymerase chain reaction (PCR) amplification and allelic discrimination plate read and analysis. PCR amplification required four components:

- 1) purified genomic DNA (20ng).
- 2) 20X SNP Genotyping Assay Mix (0.75µl), which contained sequence-specific forward and reverse primers to amplify the desired SNP and two TaqMan® minor groove binder (MGB) probes. Each probe had a reporter dye attached to its 5' end and a nonfluorescent quencher (NFQ) to its 3' end (**Figure 2.1**). One probe was labeled with VIC dye and detected the Allele 1 sequence and the other probe was labeled with FAM dye and detected the Allele 2 sequence.
- 3) 2X TaqMan® Universal PCR Master Mix, No AmpErase® Uracil-N-Glycosylase (UNG) (7.5µl), which contained AmpliTaq Gold® DNA polymerase and other components required for the PCR reaction, such as deoxynucleotides and magnesium chloride.
- 4) DNase, RNase Free, distilled water (2.75µl).

The PCR reaction was performed on an Eppendorf Mastercycler and consisted of five steps: 1) 2 minutes at 50°C, 2) 10 minutes at 95 °C, 3) 15 seconds at 95 °C, 4) 1 minute at 60 °C, 5) 40 cycles of steps 3-4. During the PCR reaction, each probe bound to a complementary sequence between the forward and reverse primers, and the proximity of the reporter dye to the quencher resulted in suppression of the reporter fluorescence. The

AmpliTaq Gold® DNA polymerase only cleaved probes that were hybridized to the target, thereby separating the reporter dye from the quencher. This resulting in an increase in fluorescence by the reporter. Following PCR amplification, allelic discrimination and analysis was performed using an ABI Prism 7000® sequence detection system (SDS). The SDS software calculated the fluorescence measurements from the plate read and created a plot of the signals. If there was a substantial increase in the VIC dye fluorescence only, it indicated that the sample was homozygous for allele 1. If there was a substantial increase in the FAM dye fluorescence only, it indicated that the sample was homozygous for allele 2. If there was a substantial increase in both fluorescent signals, it indicated heterozygosity for allele 1 and allele 2 (**Figure 2.2**).

To evaluate the quality of genotyping, 5% of the samples were randomly selected and re-genotyped. A total of 4011 SNP genotyping assays (3 SNPs × 1273 subjects + 5% verification) were completed.

2c.3: Candidate gene – Perilipin.

The three SNPs in the *PLIN* gene that were investigated were an A/C polymorphism located in the 3' untranslated region (public ID, dbSNP:rs4932241; public location: Chromosome 15 – 88005658), an A/G SNP located between the 6th and 7th exon (public ID, dbSNP: rs894160; public location: Chromosome 15 –88012827) and an A/G SNP located between the 2nd and 3rd exon (public ID, dbSNP: rs2289487; public location: Chromosome 15 – 88018100) (**Figure 2.3**).

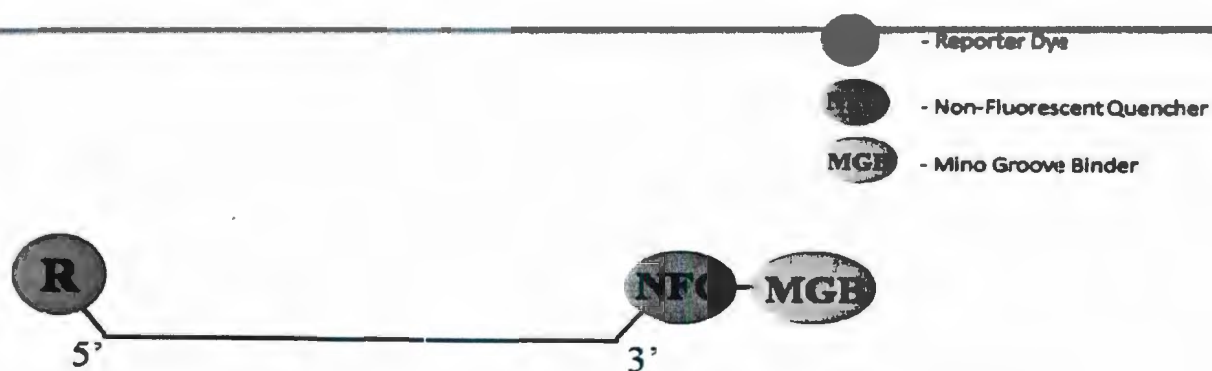


Figure 2.1: Illustration of the TaqMan minor groove binder probe with a reporter dye attached to its 5' end and a non-fluorescent quencher attached to its 3' end (adapted from ABI Assays-on-Demand Gene Expression Products Protocol).

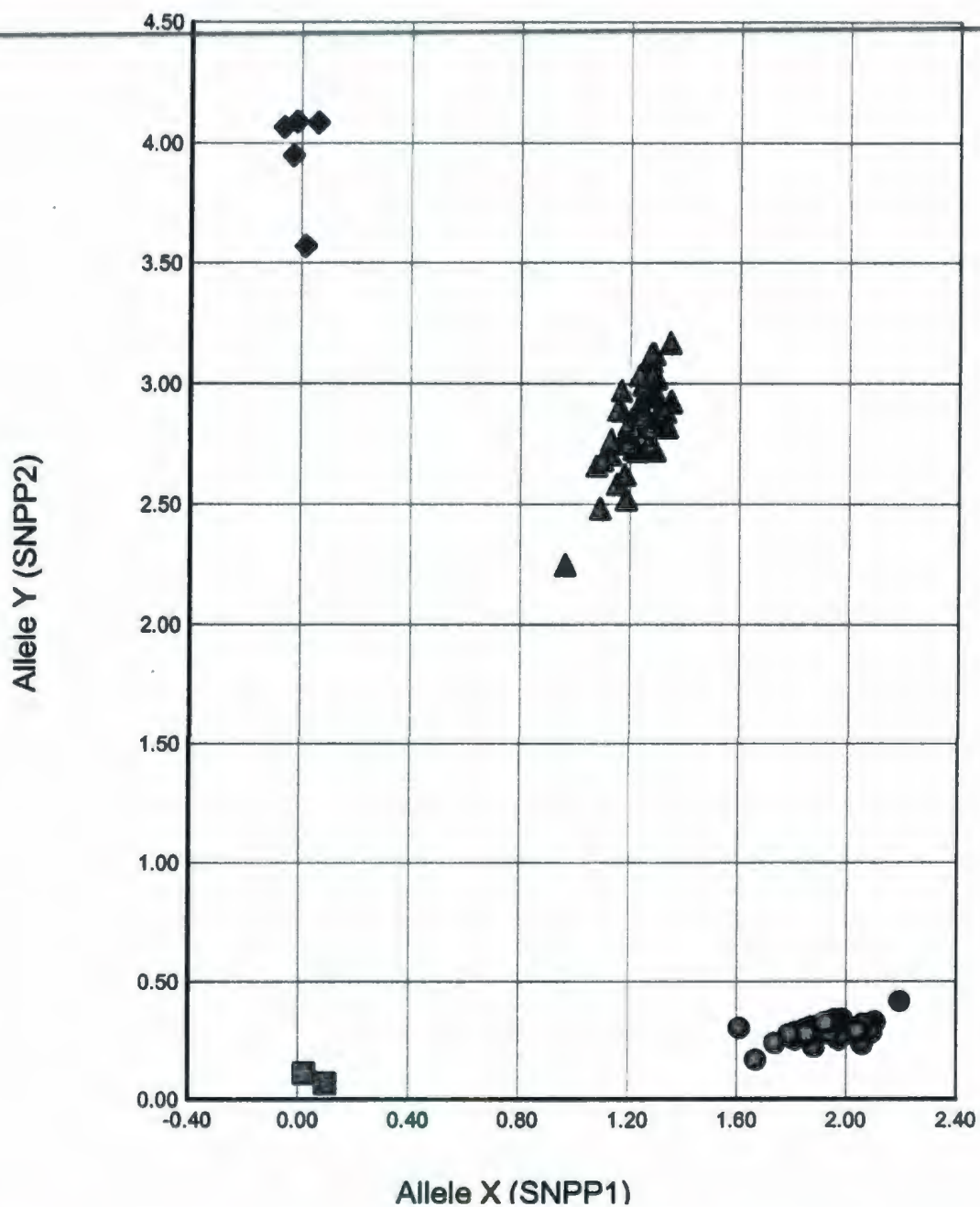


Figure 2.2: An example of an allelic discrimination showing 4 distinct groups: grey squares refer to non-template controls; blue diamonds refer to homozygosity for allele Y; green triangles refer to heterozygosity; red circles refer to homozygosity for allele X.

2c.4: Candidate gene – Adipophilin.

The two SNPs in the *ADFP* gene that were investigated were an A/G polymorphism located in the 5' UTR (public ID, dbSNP: rs3824369; public location: Chromosome 9 – 19,116,565), and an A/G SNP located in the 3' untranslated region (public ID, dbSNP: rs35629534; public location: Chromosome 9 –19,105,720) (**Figure 2.4**).

2c.5: Statistical analysis

All statistical analyses were performed using the statistical software R or SPSS software version 16 for Windows; with significance set at $p < 0.05$. Hardy-Weinberg equilibrium was tested using chi-square analysis. Chi-square analysis was also used to assess the allele differences between obese and non-obese subjects for each of the SNPs investigated. One-way ANOVA, corrected with Bonferroni *t*-test, was used to assess differences among genotype groups of SNPs with body compositions (%BF, %TF, %LF, and BMI) in males and females. Analyses were repeated after adjusting %BF, %TF, %LF, and BMI for gender and age. One-way ANOVA, corrected with Bonferroni *t*-test, was also used to assess differences among genotype groups of SNPs with serum lipid parameters in males and females. Due to the skewed distributions, the TAG parameters were transformed using \log_{10} prior to data analysis. Similarly, analyses were repeated after adjusting serum lipid parameters for age and %BF.

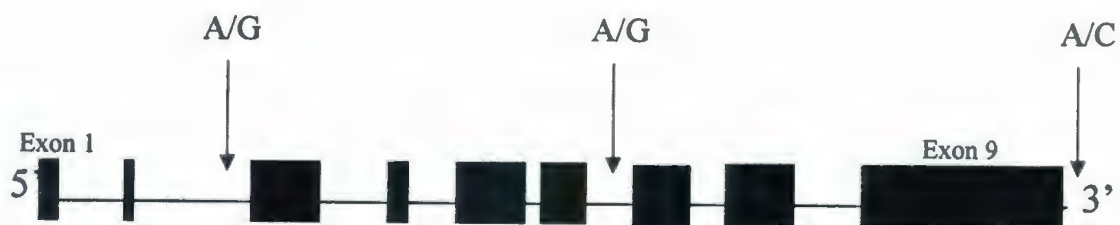


Figure 2.3: Approximate locations of the 3 SNPs investigated within the *PLIN* gene.



Figure 2.4: Approximate locations of the 2 SNPs investigated within the *ADFP* gene.

3a: Comparison of the classification of obesity by BMI versus dual-energy x-ray absorptiometry.**3a.1: Subjects**

Physical characteristics for female and male participants are shown in **Table 3.1**. The age range for all subjects ranged from 20-84 years old. Male subjects were 3.5 years younger than women on average. Men were also 16.4 kg heavier and 13.4 cm taller compared to women and had BMI measurements 1.5 units higher which reflects averages seen in similar studies [158]. Men also had greater waist-to-hip ratios and lower %BF and trunk fat percentage (%TF) compared to women.

3a.2: General discrepancy analyses by gender

Significant discrepancies between BMI and %BF criteria were identified in both women and men. Of the 1321 women included in our study, BMI classified 44.4% (584) as NW while DXA classified only 29.7% (391) as NW (**Figure 3.1**). Classification of OW women was similar between the two methods (BMI 34.1% (452), DXA 31.0% (408)). Among OB women there was again a large discrepancy between the two methods. According to BMI criteria, 20.3% (269) of women in our cohort were obese however according to %BF criteria 37.1% (493) of women were obese. As a result, BMI classified 14.7% more women as normal weight and 16.8% less women as obese compared to %BF criteria determined by DXA ($p < 0.001$). A total discrepancy of 42.2% was found between the two methods in women.

Table 3.1: Physical characteristics of female and male subjects (n = 1691)¹.

Variables	Women	Men	<i>t</i> -test
	n = 1321	n = 370	p value ²
Age (yrs)	44.1 ± 10.8 (20, 84)	40.4 ± 13.8 (20, 76)	<0.001
Weight (kg)	68.7 ± 14.1 (38.8, 156.8)	84.4 ± 13.9 (53.5, 148.4)	<0.001
Height (cm)	162.1 ± 5.8 (135.0, 186.7)	175.6 ± 6.6 (157.0, 198.1)	<0.001
BMI (kg m ⁻²)	26.5 ± 5.2 (16.0, 54.3)	27.6 ± 4.5 (16.8, 50.4)	<0.001
Waist (cm)	90.6 ± 14.3 (62.7, 168.0)	97.8 ± 12.2 (63.7, 148.8)	<0.001
Hip (cm)	102.5 ± 11.6 (76.5, 172.0)	101.3 ± 9.1 (69.0, 145.1)	<0.001
Waist-to-hip ratio	0.88 ± 0.07 (0.64, 1.24)	0.96 ± 0.06 (0.74, 1.20)	<0.001
Body fat (%)	37.6 ± 7.4 (4.6, 59.9)	23.5 ± 7.5 (5.6, 47.6)	<0.001
Trunk fat (%)	38.8 ± 8.6 (3.9, 64.3)	30.0 ± 8.8 (4.7, 53.1)	<0.001

¹ All values are mean ±SD; minimum and maximum values are in parentheses.

² Significantly different from men according to Student's *t*-test (*p*<0.001).

Of the 370 men included in this study, BMI and %BF classifications were similar for NW individuals (BMI 29.8%, DXA 32.2%; **Figure 3.2**). Among OW and OB men, significant differences were evident in weight classification among the two methods. BMI categorized 45.3% of men as OW and 24.3% as OB while DXA classified only 28.3% of men as OW and 37.7% as OB. BMI classified 17% more men as OW and 13.4% less men as obese compared to %BF criteria based on DXA measurements ($p < 0.001$). A total discrepancy of 30.4% was evident between the two methods in men.

3a.3: Discrepancy analyses by age group

After separation of the female cohort into groups based on age, similar discrepancies were evident between BMI- and %BF-defined adiposity status across all four age groups (**Table 3.2**). There was a significant discrepancy between the two methods for NW and OB women across all age groups ($p < 0.001$). The discrepancy between BMI and DXA-determined %BF ranged from 12% to 19% for NW women and 13% to 23% for OB women. Women in their 30s demonstrated the largest discrepancy between BMI and %BF for both NW and OB groups. Women in their 40s demonstrated the smallest discrepancy between the two methods among the four age groups. The discrepancies found in the female cohort for UW and OW BMI classifications compared to %BF were not significant for all age groupings. Males were not analyzed by age group due to small sample size.

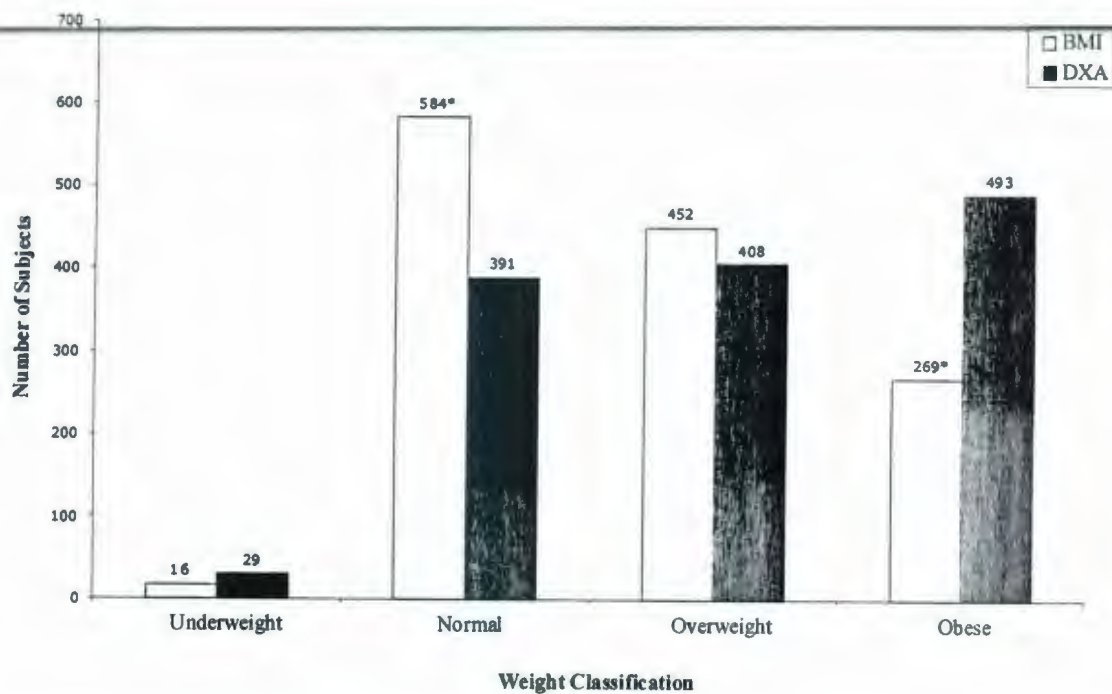


Figure 3.1: Comparison of BMI- and DXA-defined weight classifications in women according to body size (n = 1321).

* $p < 0.001$ for difference between the two methods according to Chi Square analyses.

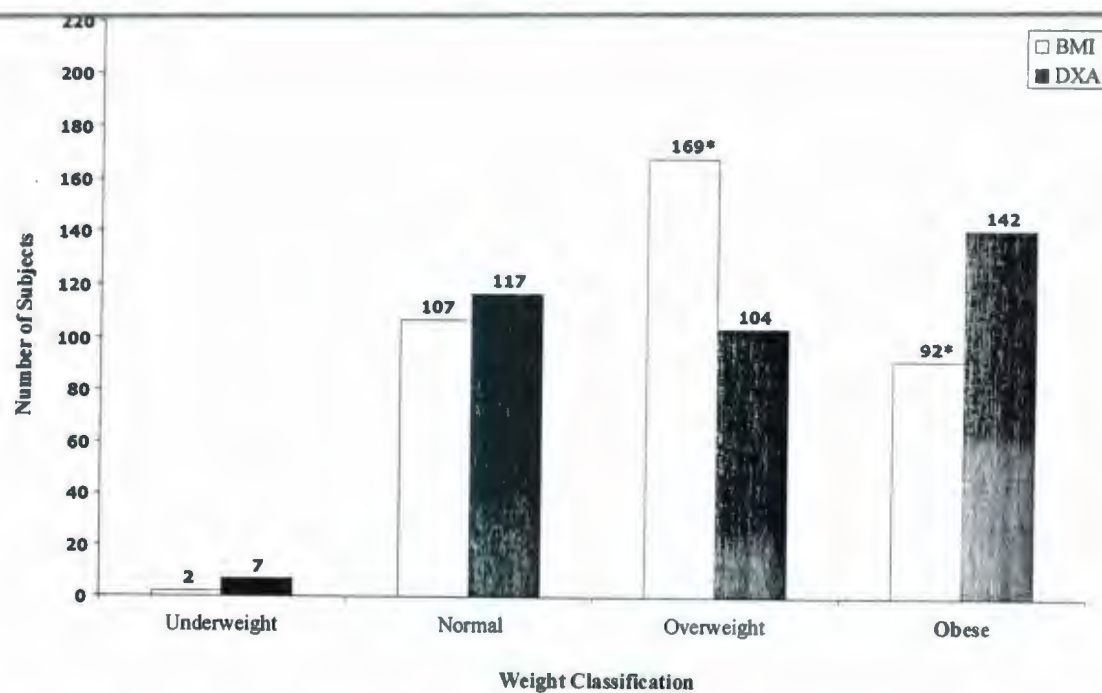


Figure 3.2: Comparison of BMI- and DXA-defined weight classifications in men according to body size (n = 370).

* $p < 0.001$ for difference between the two methods according to Chi Square analyses.

Table 3.2: Percent discrepancies between BMI and DXA weight classifications in women according to age (n = 1321)¹.

	Age Group														
	20-29.9			30-39.9			40-49.9			50+			Overall		
	n = 164			n = 204			n = 513			n = 440			n = 1321		
	BMI	DXA	%D ²	BMI	DXA	%D	BMI	DXA	%D	BMI	DXA	%D	BMI	DXA	%D
Underweight	4	9	-3	4	3	+1	5	13	-2	3	4	0	16	29	-1
Normal weight	100	69	+19*	100	63	+19*	214	155	+12*	170	104	+15*	591	395	+15*
Overweight	43	35	+5	64	56	+4	187	170	+3	158	147	+3	453	412	+1
Obese	17	51	-21*	36	82	-23*	107	175	-13*	109	185	-17*	270	494	-17*

¹ BMI and DXA values are raw numbers of women classified into each adiposity group.

² %D, Percent discrepancy between BMI- and DXA-defined weight classification; calculated as percentage of women grouped into

BMI category subtracted from percentage of women grouped into DXA category.

*Significant difference between BMI- and DXA-defined weight classification according to Chi Square analyses (p < 0.001).

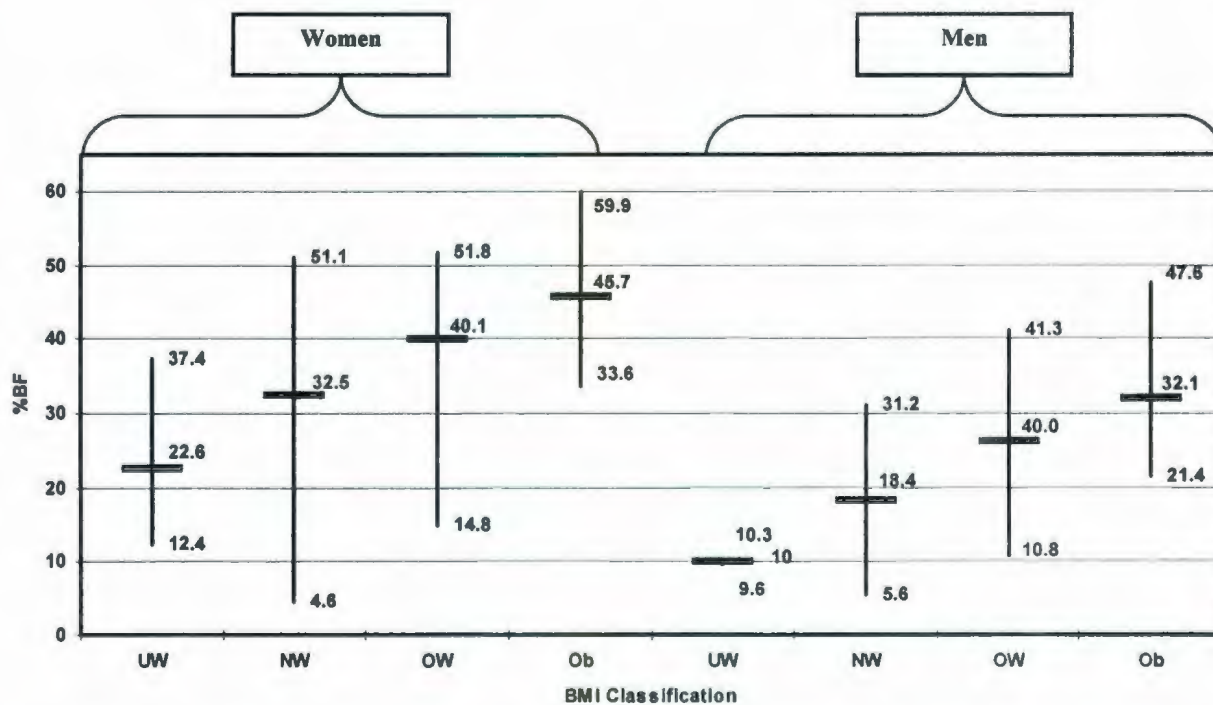


Figure 3.3: Percentage body fat (%BF) variations among women and men according to BMI classification.

%BF, percentage body fat; UW, underweight; NW, normal weight; OW, overweight; Ob, obese.

Data is presented as mean and minimum/maximum values for %BF.

3a.4. Error range in classification by BMI

The variation in %BF according to BMI categories for men and women is found in **Figure 3.3**. A large range of error indexed by %BF was found in each BMI category for both genders. A total of 224 OB women (determined by DXA) were misclassified as either NW or OW by BMI criteria. There was a wide range in %BF for NW and OW women (4.6 – 51.1% and 14.8 – 51.8%, respectively). OW women (DXA) were also misclassified as UW and NW according to BMI criteria. In addition, NW and UW women (DXA) were misclassified as OW and NW, respectively. This suggests that BMI misclassifies female subjects across all four weight classifications. The data among men was similar. A total of 50 OB men (determined by DXA) were misclassified as NW or OW according to BMI criteria. The range in %BF for NW and OW men was 5.6 – 31.2 % and 10.8 – 41.3%, respectively. Although the misclassifications were bi-directional, BMI tended to under-classify subjects for the majority of individuals.

3b: Abnormality of serum lipids are independently associated with increased serum calcium levels.

3b.1: Subjects

The physical characteristics and biochemical measurements of the adult population studied are shown in **Table 3.3**. There are significant differences between women and men in all physical characteristics and biochemical markers except for total serum calcium concentration corrected for serum albumin (serum $\text{Ca}^{++}\text{-C}$), Mg^{++} and LDL-c.

3b.2: Partial correlations between serum $\text{Ca}^{++}\text{-C}$ concentration and lipids

The results of the partial correlations between serum $\text{Ca}^{++}\text{-C}$ and lipid markers based on gender are shown in **Table 3.4**. Significant positive correlations of serum total cholesterol, TAG, HDL-c and LDL-c with serum $\text{Ca}^{++}\text{-C}$ were found in women ($r = 0.26$, $r = 0.16$, $r = 0.11$ and $r = 0.19$, respectively; $p < 0.0001$ for all variables). Significant correlations between serum $\text{Ca}^{++}\text{-C}$ and serum total cholesterol, HDL-c and LDL-c were also observed for men ($r = 0.13$, $r = 0.12$ and $r = 0.10$, respectively; $p = 0.05$ to 0.009).

Women and men not taking any medication were analyzed using partial correlation, controlling for age, trunk fat, phosphorus and magnesium (**Table 3.5**). The significant positive correlations remained between serum $\text{Ca}^{++}\text{-C}$ and lipids in women, but the significant HDL-c correlation was lost in men.

Women were further categorized into pre- and post-menopausal groups for analyses in **Table 3.6**. The significant positive correlations between serum $\text{Ca}^{++}\text{-C}$ and all four lipid markers remained in both pre- and post-menopausal women groups.

Table 3.3: Physical characteristics and biochemical measurements of subjects.

	Females (n=1383 -1472)	Males (n=422 -435)
Age (years)	44.7 ± 11.3 (19 -84)	41.0 ± 13.3 (19 -82)
Weight (kg)	69.6 ± 14.1 (39.5 -156.8)	85.0 ± 14.2 (53.6 -149.4)
Height (cm)	162.1 ± 5.8 (135.0 -186.7)	175.7 ± 6.3 (157 -198)
BMI (kg/m ²)	26.5 ± 5.3 (16.0 -54.3)	27.6 ± 4.4 (16.8 -50.4)
Waist circumference (cm)	90.9 ± 14.4 (54 -168)	97.9 ± 11.9 (64 -149)
%FAT	37.8 ± 7.5 (4.6 -60.2)	25.3 ± 7.5 (5.6 -48.0)
%FAT-Trunk	38.9 ± 8.8 (3.9 -65.3)	30.0 ± 8.8 (4.7 -53.0)
Lean body mass (kg)	39.7 ± 5.5 (25.8 -81.9)	59.5 ± 7.5 (37.2 -100.5)
Serum Ca ⁺⁺ (mmol/L)	2.34 ± 0.12 (1.72-3.01)	2.36 ± 0.12 (1.76 -2.81)
Serum Ca ⁺⁺ -C (mmol/L) * ¹ ,* ²	2.33 ± 0.11 (1.67 -2.92)	2.32 ± 0.10 (1.91 -2.68)
Serum Phosphorus (mmol/L)	1.21 ± 0.17 (0.69 -1.97)	1.13 ± 0.18 (0.65 -1.66)
Serum Mg ⁺⁺ (mmol/L)* ¹	0.88 ± 0.08 (0.47 -1.48)	0.89 ± 0.11 (0.11 -1.85)
Cholesterol (mmol/L)	5.21 ± 1.03 (2.40 -9.98)	5.02 ± 0.97 (2.82 -6.97)
Triglyceride (mmo l/L)	1.15 ± 0.70 (0.23 -5.88)	1.24 ± 0.71 (0.35 -3.75)
HDL -cholesterol (mmol/L)	1.56 ± 0.38 (0.77 -3.25)	1.40 ± 0.36 (0.65 -2.34)
LDL-cholesterol (mmol/L) * ¹	3.12 ± 0.89 (0.65 -7.61)	3.06 ± 0.84 (1.31 -5.00)
Risk factor	3.48 ± 0.95 (0.68 -8.41)	4.22 ± 1.16 (2.08 -8.51)

Data are means ± SD;

*¹ p value < 0.05-0.001 for all variables, except for serum Ca⁺⁺-C, Mg⁺⁺ and LDL-cholesterol in the comparison between women and men using Student's *t*-test;

*² Calcium-C means total serum calcium corrected by albumin (Ca+0.1 x (40-albumin)/6).

Table 3.4: Partial correlations between serum Ca^{++} -C concentration and lipids.

Parameter	Females (n=1373)		Males (n=382)	
	r	p	r	p
Cholesterol (mmol/L)	0.26	<0.0001	0.13	0.009
Triglyceride (mmol/L)	0.16	<0.0001	0.05	ns
HDL (mmol/L)	0.11	<0.0001	0.12	0.016
LDL (mmol/L)	0.19	<0.0001	0.10	0.05

Confounding factors including age, trunk fat percentage, phosphorus and magnesium were correspondingly controlled in analyses where applicable.

Table 3.5: Partial correlations between serum Ca^{++} -C concentration and lipids in subjects without taking any medication.

Parameter	Females (n=595)		Males (n=236)	
	r	p	r	p
Cholesterol (mmol/L)	0.24	<0.0001	0.19	0.004
Triglyceride (mmol/L)	0.09	0.028	0.12	0.056
HDL (mmol/L)	0.12	0.005	0.10	ns
LDL (mmol/L)	0.19	<0.0001	0.16	0.015

Confounding factors including age, trunk fat percentage, phosphorus and magnesium were correspondingly controlled in analyses where applicable.

Table 3.6: Partial correlations between serum Ca^{++} -C concentration and lipids in pre- and post-menopausal women.

Parameter	Pre-menopausal (n=763)		Post-menopausal (n=595)	
	r	p	r	p
Cholesterol (mmol/L)	0.31	<0.0001	0.24	<0.0001
Triglyceride (mmol/L)	0.16	<0.0001	0.09	<0.05
HDL (mmol/L)	0.17	<0.0001	0.12	0.005
LDL (mmol/L)	0.21	<0.0001	0.19	<0.0001

Confounding factors including age, trunk fat percentage, phosphorus and magnesium were correspondingly controlled in analyses where applicable.

Table 3.7: Levels of serum cholesterol, TAG, HDL-c and LDL-c among low, medium and high serum Ca^{2+} -C groups.

Calcium group		Women	Men
		n=1378-1381	n=386-389
Cholesterol (mmol/l)	L	4.97 ± 0.88	5.01 ± 1.03
	M	5.10 ± 1.02	5.11 ± 1.13
	H	5.56 ± 1.09	5.17 ± 1.22
P value		<0.0001*	ns
TAG (mmol/L)	L	1.03 ± 0.61	1.80 ± 0.91
	M	1.07 ± 0.61	1.54 ± 1.08
	H	1.32 ± 0.83	1.48 ± 1.11
P value		<0.0001* ¹	ns
HDL-c (mmol/L)	L	1.53 ± 0.35	1.20 ± 0.24
	M	1.56 ± 0.37	1.22 ± 0.27
	H	1.60 ± 0.41	1.28 ± 0.28
P value		0.017* ²	<0.05* ²
LDL-c (mmol/L)	L	2.96 ± 0.78	3.13 ± 0.88
	M	3.06 ± 0.88	3.19 ± 0.95
	H	3.36 ± 0.94	3.22 ± 0.94
P value		<0.0001* ¹	ns

Data are means ± SD. Significance assessed by one-way ANOVA, corrected by TUKEY method. * Significance between any two groups; *¹ Significance between L vs H, and M vs H; *² Significance between L vs H. L= low calcium group; M=medium calcium group; H= high calcium group.

3b.3: Analysis for the effects of covariates on serum calcium, cholesterol, TAG, HDL-c and LDL-c

The levels of serum lipids in female and male subjects according to the concentrations of serum Ca^{++} -C are shown in (Table 3.7). In women, those in the low serum Ca^{++} -C group had the lowest fasting total cholesterol, HDL-c and LDL-c levels, those in the medium serum Ca^{++} -C group had an intermediate total cholesterol, HDL-c and LDL-c levels, and those in the high calcium group had the highest total cholesterol, TAG, HDL-c and LDL-c levels, ($P < 0.0001$ for all four variables). The only significant difference between calcium groups found in men was with HDL-c.

Moreover, the effects of 25-OH vitamin D and PTH were adjusted in a selected group of 100 women (Table 3.8). Subjects with low serum Ca^{++} -C concentrations had a significantly lower level of fasting total cholesterol, TAG and LDL-c compared with subjects with high calcium concentration ($P < 0.0001$, $=0.015$ and $=0.004$, respectively).

Table 3.8: Comparisons of lipids between low and high calcium groups adjusted for 25-OH vitamin D and PTH in female subjects.

	Low calcium group (n=50)	High calcium group (n=50)	p
Cholesterol (mmol/L)	4.83 ± 0.12	5.83 ± 0.17	<0.0001
TAG (mmol/L)	0.97 ± 0.08	1.40 ± 0.13	0.015
HDL-c (mmol/L)	1.54 ± 0.05	1.67 ± 0.06	ns
LDL-c (mmol/L)	2.85 ± 0.10	3.53 ± 0.15	0.004

Student *t*-test. Values are shown in mean ± SE of raw values. Before analysis, total serum calcium was adjusted for 25-OH vitamin D and PTH, serum cholesterol, TAG, HDL-c and LDL-c for age and %trunk fat using linear regression analysis.

Table 3.9: Demographic and body composition data for all subjects. (n=1269)

	Female (n=1002)	Male (n=267)	Overall (n=1269)	t-test (p-value)
Age (y)	43 ± 10.0 (19, 62)	39.8 ± 12.7 (19, 61)	42.2 ± 10.7 (19, 62)	<0.0001
Weight (Kg)	69.1 ± 13.8 (45.4, 157)	85.1 ± 15.0 (53.6, 149.4)	72.5 ± 15.5 (45.4, 156.8)	<0.0001
Height (cm)	162. ± 5.9 (135, 191)	175.2 ± 6.6 (157, 198)	164.9 ± 8.1 (135, 198)	<0.0001
BMI (Kg/m ²)	26.3 ± 5.1 (17, 54.3)	27.8 ± 4.7 (16.8, 50.4)	26.6 ± 5.0 (16.8, 54.3)	<0.0001
Waist (cm)	90.1 ± 13.9 (63.6, 168)	98.1 ± 12.5 (63.7, 148.8)	91.8 ± 14.0 (63.6, 168)	<0.0001
Hip (cm)	102 ± 11.3 (76.5, 172)	101.5 ± 9.4 (76.5, 145.1)	102.1 ± 10.9 (76.5, 172)	<0.0001
W/H Ratio	0.88 ± 0.07 (0.6, 1.2)	0.97 ± 0.06 (0.74, 1.2)	0.90 ± 0.08 (0.6, 1.2)	<0.0001
%BF	37.3 ± 7.1 (4.6, 59.9)	25.7 ± 7.4 (7.4, 47.6)	34.9 ± 8.6 (4.6, 59.9)	<0.0001
%TF	38.4 ± 8.2 (3.9, 64.3)	30.4 ± 8.5 (8.7, 53.1)	36.7 ± 8.9 (3.9, 64.3)	<0.0001
Cholesterol	5.25 ± 1.0 (2.3, 10)	5.09 ± 1.1 (2.8, 9.2)	5.21 ± 1.0 (2.3, 10)	<0.0001
HDL-c	1.58 ± 0.36 (0.8, 3.3)	1.26 ± 0.25 (0.7, 2.2)	1.51 ± 0.36 (0.7, 3.3)	<0.0001
LDL-c	3.13 ± 0.89 (0.7, 7.6)	3.15 ± 0.87 (0.7, 5.7)	3.13 ± 0.89 (0.7, 7.6)	<0.0001
TG	1.17 ± 0.73 (0.2, 5.9)	1.50 ± 1.0 (0.3, 5.0)	1.24 ± 0.8 (0.2, 5.9)	<0.0001

3c: Genetic association study – Roles of Perilipin and Adipophilin on obesity phenotypes.

3c.1: Subjects

The physical and biochemical characteristics of the subjects are shown in **Table 3.9**. There were 1269 eligible subjects (1002 females and 267 males). The subjects' ages ranged from 19-62 years old. Male subjects were 3.2 years younger than women on average. Men were also 16 kg heavier, 13.2 cm taller, had BMI measurements 1.5 units higher and had higher serum cholesterol and TAG measures, and waist-to-hip ratios greater than the female cohort. Women had greater %BF and trunk fat percentage (%TF), higher serum HDL-c and total serum cholesterol compared to men. These population characteristics reflect averages seen in similar studies [158].

A summary of the 5 SNPs investigated including allele frequencies, allelic variation and location are presented in **Table 3.10**. SNPs rs4932241 (3'UTR), rs894160 (between exon 6 and 7) and rs2289487 (between exon 2 and 3) are located in the perilipin coding region on chromosome 15. The frequencies for alleles A and C of the SNP rs4932241 were 0.69 and 0.31, frequencies for alleles A and G for the SNP rs894160 were 0.74 and 0.26, and frequencies for alleles A and G of the SNP rs2289487 were 0.68 and 0.32, respectively (**Table 3.10A**).

SNPs rs3824369 (5'UTR) and rs35629534 (3'UTR) are located within the adipophilin coding region on chromosome 9. The frequencies for alleles A and G of the SNP rs3824369 were 0.95 and 0.05, and the frequencies for alleles A and G for the SNP rs35629534 were 0.51 and 0.49 (**Table 3.10B**). All SNPs investigated were in Hardy-

Weinberg equilibrium according to Chi-Square analysis. Linkage disequilibrium was also estimated for the SNPs investigated and is presented in **Table 3.11**.

Power calculations were carried out for a quantitative trait genetic association study [159]. The causal locus was reflected indirectly by rs4932241 and type 1 error was fixed to 10^{-6} . **Figure 3.4** shows the power profiles as a function of varying coefficients of determination for a range of heritability estimates ($h^2 = 0.05-0.15$). As demonstrated by this figure, there was sufficient power ($\beta \geq 0.80$) at $R^2 > 0.4$, given our sample size, to detect a positive association.

Table 3.10. Summary of single nucleotide polymorphisms, allele frequencies, and Hardy-Weinberg equilibrium^a SNPs from PLIN(A) and ADFP(B).

A

RS Number	Variant	Location	Frequency	HWE (<i>p</i> -value)
rs4932241 ^b	A/C	3'UTR	0.69 / 0.31	0.468
rs894160 ^b	A/G	Intron 6	0.74 / 0.26	0.508
rs2289487 ^b	C/A	Intron 2	0.68 / 0.32	0.896

^aHardy-Weingberg was estimated using using χ^2 analysis.

^bSNPs from the *PLIN* gene.

B

RS Number	Variant	Location	Frequency	HWE (<i>p</i> -value)
rs3824369 ^c	A/G	5'UTR	0.95 / 0.05	0.531
rs35629534 ^c	A/G	3'UTR	0.51 / 0.49	0.910

^aHardy-Weingberg was estimated using using χ^2 analysis.

^cSNPs from the *ADFP* gene.

Table 3.11: Estimated pairwise linkage disequilibrium and sample size.

N/D'	rs4932241	rs894160	rs2289487	rs3824369	rs35629534
rs4932241		$< 10^{-16}$	$< 10^{-16}$	0.151	0.777
rs894160	1230		$< 10^{-16}$	0.189	0.801
rs2289487	1255	1231		0.556	0.413
rs3824369	1256	1232	1257		$< 10^{-16}$
rs35629534	1245	1220	1245	1246	

The genotype effect of the three SNPs analyzed from the *PLIN* locus and the two SNPs analyzed from the *ADFP* locus on markers of obesity and serum lipids are found in **Table 3.12**. We tested for associations between the five SNPs and %BF, cholesterol, HDL-c, LDL-c, and TAG using multiple regression analyses with gender and age as model covariates. No significant association was found between the three perilipin SNPs tested and %BF or with serum lipids. No significant association was found between the two *ADFP* SNPs analyzed and HDL-c or TAG. In addition, no significance was found between *ADFP* SNP rs35629534 and cholesterol, LDL-c or %BF. A significant association was found between *ADFP* SNP rs3824369 and cholesterol, LDL-c and %BF. Carriers of the major allele of rs3824369 had significantly higher fasting levels of LDL-c (**Table 3.13**). Carriers of the major allele of rs3824369 had significantly higher fasting levels of cholesterol compared to homozygote carriers of the minor allele (**Table 3.14**). In addition, carriers of the major allele of SNP rs3824369 had significantly higher %BF compared to homozygote carriers of the minor allele (**Table 3.15**). To our knowledge this

is the first report of an association between a SNP in *ADFP* and variations in body fat or serum lipids in an adult population.

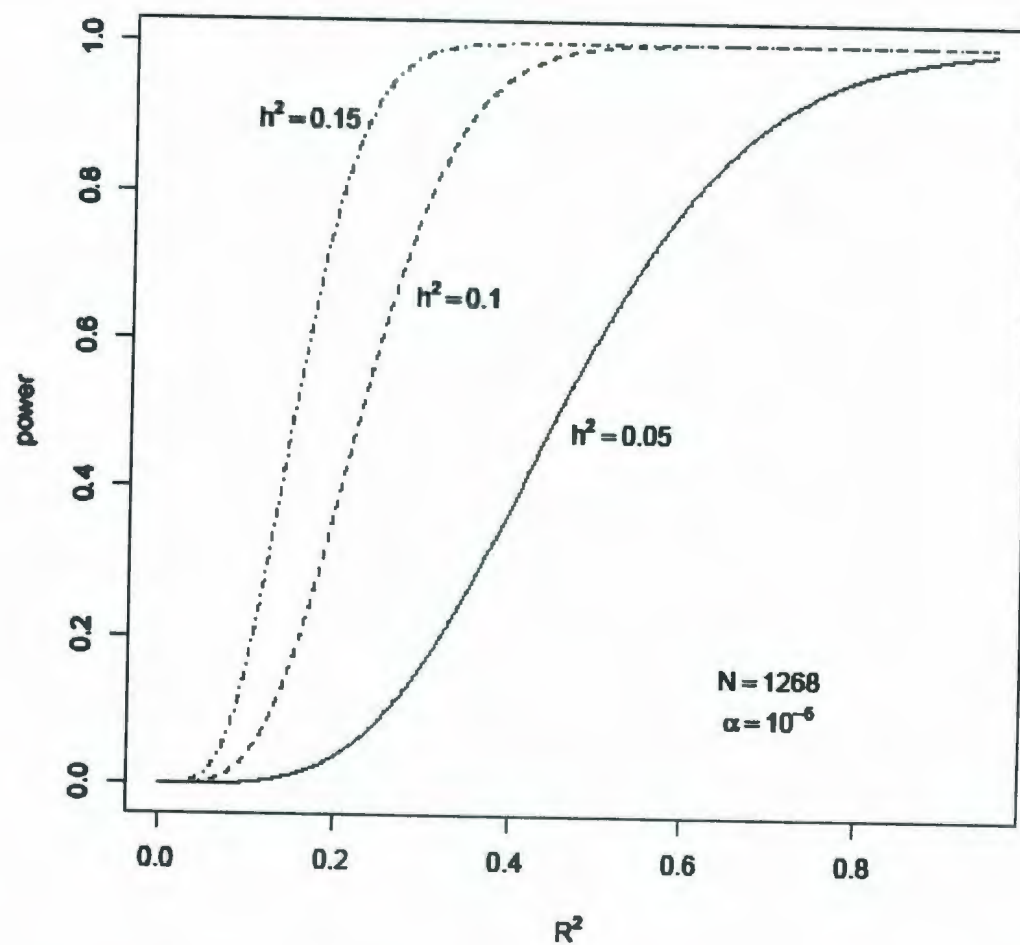


Figure 3.4. Power profiles as a function of varying coefficients of determination for a range of heritability estimates.

Table 3.12 Genotype effect of three SNPs within *PLIN* and two SNPs within *ADFP* on markers of obesity and serum lipids.

	rs4932241 ^b	rs894160 ^b	rs2289487 ^b	rs3824369 ^c	rs35629534 ^c
Variables	<i>P</i> values ^a				
Cholesterol					
(mmol/l)	1.000	1.000	1.000	0.004	0.381
HDL-C (mmol/l)	1.000	1.000	1.000	1.000	1.000
LDL-C (mmol/l)	1.000	1.000	1.000	0.007	1.000
Triacylglycerol					
(mmol/l)	0.996	1.000	1.000	1.000	1.000
%BF	0.997	0.997	0.997	0.024	1.000

Multiple regression using an allele dosage or additive model were used for quantitative trait analyses to assess the association between *PLIN* variants and markers of obesity and serum lipids. Sex and age were included as model covariates.

HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; *PLIN*, Perilipin;

^a*P* values based on 10,000 permutations.

^bSNPs from the *PLIN* gene.

^cSNPs from the *ADFP* gene.

Table 3.13: Serum LDL-c levels according to *ADFP* genotype.

Genotype	n	LDL-c (mmol/l)
rs3824369		
AA	1146	3.12 ± 0.87
AG	116	3.29 ± 1.07
GG	4	2.64 ± 1.15

Table 3.14: Serum cholesterol levels according to *ADFP* genotype.

Genotype	n	Cholesterol (mmol/l)
rs3824369		
AA	1146	5.20 ± 1.01
AG	116	5.35 ± 1.22
GG	4	4.54 ± 1.46

Table 3.15: DXA measured percentage body fat according to *ADFP* genotype.

Genotype	n	%BF
rs3824369		
AA	1146	34.8 ± 8.6
AG	116	35.0 ± 8.6
GG	4	33.2 ± 9.5

4: Discussion:

4a: Comparison of the classification of obesity by BMI versus dual-energy x-ray absorptiometry.

Our study, involving a large sample from the Newfoundland population, demonstrates the limited ability of BMI to accurately estimate adiposity. We have revealed a large discrepancy between BMI- and DXA-defined adiposity status that is both gender and age specific. Almost one half of women and one third of men were misclassified by BMI criteria compared to %BF criteria determined by DXA. A significant proportion of obese individuals were classified as either normal weight or overweight according to BMI criteria. This poses serious health consequences on a population level as the opportunity to intervene and reduce health risk in these individuals is lost. Overall, BMI had the poorest ability to predict true adiposity in normal weight and obese women, and in overweight and obese men. Furthermore, this misclassification was influenced by age, with younger women (under 40 years old) demonstrating the largest discrepancy between the two methods. We also found significant inter-subject variability in %BF for any given BMI value.

The idea that BMI is imprecise is not a new concept. It has previously been shown that BMI is not accurate at predicting adiposity status in the normal to mildly obese range [11, 160-161] as well as in severely obese individuals [162]. In particular, BMI was found not to be accurate at predicting obesity in individuals with a body mass less than 80 kg compared to %BF determined by DXA [160]. Similarly, a significant number of people with a BMI below 30 were actually obese when classified by %BF determined by Bioelectric Impedance Analysis (BIA) [11]. A more recent study, involving a large multiethnic sample from the US population found BMI to have limited

diagnostic performance, especially in those with a BMI < 30 [161]. Despite BMI-defined obesity having good specificity when compared to BIA-defined obesity, BMI had low sensitivity, missing nearly half of %BF-determined obese people. These findings suggest that BMI may not be accurate at assessing adiposity status in normal weight and overweight individuals. Our study included all ranges of BMI and %BF (16.0 – 54.3 kg.m⁻² and 4.6 - 59.9 %, respectively) and revealed a slightly higher discrepancy.

We observed gender differences in the discrepancy between the two methods. Although there was good agreement between BMI and DXA for overweight women, BMI had limited ability to predict the correct weight classification for normal weight and obese women. In men, the greatest discrepancy was evident in the overweight and obese groups. Gender differences in BMI accuracy have previously been shown. In men, BMI correlates better with lean mass compared to %BF however in women BMI correlates better with %BF [161]. This may explain why there was a greater discrepancy between BMI and %BF-defined adiposity status in overweight men but not in women. Furthermore, the association between BMI and %BF differs between males and females. Males demonstrate a linear relationship between these two parameters while females demonstrate a curvilinear relationship [162] which may explain why we observed a high discrepancy between BMI- and DXA-defined normal weight women but not men. Gender differences in body composition are a profound physiological phenomenon however standard WHO BMI criteria do not accommodate for this. Our results suggest that this problem needs addressing. A re-adjustment of obesity criteria to include accommodations for gender differences will increase the accuracy of BMI to predict adiposity in both males and females.

We also analyzed our data after stratifying females according to age groups. The largest discrepancy between BMI and DXA weight classifications was evident in women under the age of 40 while there was moderate to good agreement between the two methods in older women. These results are surprising as previous studies have found that the diagnostic performance of BMI diminishes as age increases [161], likely due to an increase in the ratio of fat mass to fat free mass that is evident with age [163]. Further studies are warranted to address the potential mechanism surrounding this phenomenon. Obesity criteria based on %BF are age specific however, BMI criteria are identical across all age groups. From our results it is apparent that BMI cannot accurately reflect age-related changes in adiposity.

Our analysis was originally performed using %BF criteria from earlier publications by Dr. Bray. Bray's original obesity criteria (defined as $BF > 25\%$ in men and $BF > 33\%$ in women) lacked any adjustment for age or ethnicity [164]. Using these criteria, we found that approximately 72% of obese females and 54% of obese males were misclassified as normal weight or overweight according to BMI criteria. Our current results indicate that the new Bray body fat classifications (Table 1.1) [30] are a better fit to BMI criteria, however a significant margin of error still remains between the two methods. It is evident that age, gender and ethnicity-specific criteria are necessary for more accurate BMI calculations that reflect %BF.

The findings from our study highlight the importance of using caution when defining adiposity status using BMI criteria. Although previous studies have demonstrated similar trends, most have small sample sizes [19, 165-166] or have used less accurate methods to estimate %BF such as BIA or skin fold thickness [11, 161, 167].

To the best of our knowledge, this is the first study of its kind to demonstrate a discrepancy between BMI- and DXA- defined adiposity in a large cohort containing both men and women of all different age groups. Nevertheless, our study is not without limitations. Other methods to measure adiposity, such as BIA, are cheaper and easier to use, despite their reported limitations. Our study was also limited in the number of male participants and ethnic groups. Future studies investigating the discrepancy between BMI- and DXA-defined adiposity are warranted in a larger male cohort and in other populations.

In summary, we compared BMI weight classifications to DXA-determined weight classifications based on %BF in 1691 adult Newfoundlanders. BMI misclassified 42% of women and 32.5% of men into an incorrect obesity category. The misclassification was gender specific. The largest discrepancy was seen in normal weight and obese women, and in overweight men. BMI misclassifications were also influenced by age, with the largest discrepancy observed in women under 40 years old. These findings support previous research suggesting revisions of current BMI criteria to include such confounding factors as age, gender and ethnicity [162, 168-169]. Further research is needed to help alleviate these problems so that BMI can continue to be used in everyday health appraisals. Using the current BMI criteria might be inappropriate as it may misdiagnose obese individuals as normal weight. For these reasons, we recommend that caution should be taken when BMI is used in clinical and scientific research.

4b: Abnormality of serum lipids are independently associated with increased serum calcium levels in the adult Newfoundland population.

The regulation of serum calcium and the metabolism of lipids involve many hormonal and physiological factors. Factors such as age, gender, physical activity level, and body composition are contributors to variations of serum lipids seen in the general population [170-171]. Consequently, when attempting to reveal true associations between total serum calcium and any variable of serum lipids, it is critical to exclude or control as many confounding factors as possible for example, age is usually a constant non-modifiable risk factor for dyslipidemia [172-173]. Total serum cholesterol, LDL-c and TAG levels increase with age, while HDL-c levels decrease with age [172]. In the present study, age was controlled in all analytical processes.

Gender is a constant factor that affects most physiological and pathophysiological conditions [174]. For example, the liver is an important organ for lipid metabolism and significant sex differences in hepatic gene expression have been seen in mice [175]. These differences contribute to sex differences in physiology, homeostasis, and steroid and foreign compound metabolism. Sex-related differences in gene expression are common across organisms including humans [176-177]. Although most findings in our present study show similar results between men and women, sex difference in the relationship between serum calcium and TAG are evident. The positive associations were strong in women in every aspect of the analyses. However, the relatively weak associations in men might be due in part to the small sample size compared with women.

During menopause there are physiological changes that occur and the metabolism in pre- and post menopausal women can be very different. This difference can be partly due

~~to the significant change in female hormone levels and the increase of body fat associated~~
with menopause. It is usually seen that menopause is associated with potentially adverse changes in lipids and lipoproteins [178]. In our study we found that the positive associations between serum Ca^{++} and abnormalities of lipids were stronger in pre- than in post- menopausal women.

The association between some lipids and serum calcium in humans was reported in a number of cross-sectional and longitudinal studies [141, 150]. These results were documented as part of a wide range of epidemiological studies targeting general risk factors. De Bacquer and colleagues was the first group to specifically design a study to investigate the link of serum calcium with total cholesterol and HDL-c [179]. Although age, sex and BMI were considered in the analyses, other potent confounding factors, with potentially important roles in influencing either serum calcium or lipid profiles including medication use, smoking, magnesium, percent body fat and menopausal status in women, were not studied.

There is a close association between dyslipidemia and obesity, especially central obesity [180]. Central obesity is a major factor for dyslipidemia [174, 181-182]. Proper control and exclusion of the factors which influence obesity status would be critical to identifying the association between the two variables that we are interested in. A very recent study showed that DXA derived abdominal-fat mass was the best predictor of blood lipid profiles [183]. The central fat percentage measured by DXA was controlled in all analyses in our study. Control of this variable strengthened our findings, leading to the conclusion that the associations of calcium status with lipids are independent of the influences of age, gender, menopausal status and body fat.

Sedentary life style in western countries is a risk factor for dyslipidemia [182, 184].

Decreased physical activity is significantly associated with increased serum cholesterol, LDL-c and TAGs and also with decreased serum levels of HDL-c. Physical activity is therefore a potent factor affecting circulating levels of lipids. The physical activity levels of our subjects were taken into consideration in our data analyses. Importantly, the significant associations between serum calcium and lipids largely hold even after the control of physical activity levels.

The use of various medications including multi-vitamin supplements was found in nearly 50% of the study participants. This is typical of adult subjects, including the young, middle aged, and elderly, recruited from the general population. Due to the diversity and different mixes of medications used it is difficult to classify subjects based on the type of pharmaceutical effect. Medication can affect serum lipids through many mechanisms. For example, endogenous synthesis of cholesterol in the liver is controlled through modification of microsomal enzyme 3-hydroxy-3-methylglutaryl- CoA (HMG-CoA) reductase activity, the rate limiting step. Medications targeting HMG-CoA reductase such as statins can effectively lower cholesterol levels and are widely used in patients with dyslipidemia [185]. Other medications including fibric acids and nicotinic acid also have powerful lipid lowering effects and are widely used in patients with metabolic syndrome and DM2 [186]. Other drugs that are not primarily targeting lipids may still have lipid altering affects. Moreover oral contraceptives are commonly used in women of reproductive age. Oral contraceptives can cause hypertriglyceridemia in some women [187]. It is essential to exclude both the primary and the secondary influence of all types of medication on the variation of serum lipids. Analyses according to medication

~~status were therefore performed. Equally significant associations were observed in non-~~
medication users although the number of subjects was reduced by about half compared to the total group. This may indicate that the positive association between serum calcium and lipid profile becomes clearer when not confounded by the influence of medications.

Serum calcium levels are regulated by the action of PTH. PTH acts to increase the concentration of calcium in the blood [188]. PTH regulates serum calcium levels through its effects on bone, kidney and intestines. Moreover, PTH increases the activity of 1- α -hydroxylase enzyme, which converts 25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol, the active form of vitamin D. Vitamin D regulates serum calcium levels by promoting calcium absorption from food in the intestines. Blood calcium status is also augmented by re-absorption of calcium in the kidneys as well. PTH and vitamin D are the two hormones primarily involved in the regulation of serum calcium. The potential influence of these two hormones on the associations between serum calcium and lipids were excluded in this study. Moreover the interactions between serum calcium, magnesium and phosphorus may potentially modify the concentration of these cations. Hence the influence of magnesium and phosphorus was controlled in analyses in this study. Positive correlations were simultaneously observed between Ca^{++} and LDL-c, TAG and HDL-c. It seems confusing if we hypothesize that raised levels of serum Ca^{++} is the molecular basis of high LDL-c and TAG. This was an observational study and the nature of the study does not allow us to properly address this 'contradictory result'. This seemingly contradictory positive correlation with Ca^{++} could potentially be caused by numerous reasons. One of the explanations might be that the human body tries to offset the rising LDL-c and TAG by increasing the good cholesterol, HDL-c,

~~simultaneously Obviously this issue warrants further study in the future.~~ To our knowledge, this is the first study designed to investigate the relationship between the variations of lipids and serum calcium in a large population with a comprehensive control of major confounding factors. Owing to the nature of association studies, positive associations like that described in this study, cannot establish a cause-effect relationship. However, the carefully controlled study design and the strong association revealed in the analyses provide strong support for the need for later studies to examine if there is a molecular basis for the association between abnormalities of serum lipids and calcium variation.

In summary, this study analyzed the relationship between the abnormalities of serum lipids and serum calcium. A positive association was found between serum Ca^{++} -C and total cholesterol, TAG, HDL-c and LDL-c in women from the general Newfoundland population. A similar trend was found in men. Demonstrating a relationship between calcium and serum lipids in spite of comprehensive control of major confounding factors makes this study unique and further strengthens the possibility of a direct relationship.

4c: Genetic association study – roles of Perilipin and Adipophilin on obesity phenotypes.

Lipid storage droplets are the energy storehouses of most cellular organisms from fungi to mammals [189]. These sequestered lipids are used for energy, membrane components and other cellular structures. Initial research into these organelles exposed a number of proteins that appeared to regulate the building and breakdown of the lipid storage droplet [83, 86, 190-191]. Further research revealed a highly conserved family of

proteins names the PAT proteins, that all appeared to associate with lipid storage droplets in one way or another [75, 102].

Soon after the initial discovery of PAT proteins, a protein named perilipin was identified as a potential gatekeeper to the lipid stored in the adipocyte [88, 90, 93]. Depending on the metabolic needs of the organism, Perilipin can activate or inhibit the breakdown of lipid stores from lipid storage droplets.

The main role of ADFP appears to be recruitment of triacylglycerides during the initial formation of lipid droplets. This is interesting as variations in the functioning of ADFP could result in altered levels of stored lipids and of free fatty acids in the blood stream. In fact, research has shown that *ADFP* knockout mice have a reduction in liver TAG content with no affect on levels of other lipids within the liver. Chang *et al.* showed that *ADFP* knockout mice fed regular chow showed a 60% reduction in hepatic TAG [192]. With secretion of TAG in the form of lipoproteins (ex. VLDL) unaffected in *ADFP* knockout mice, it appears that the problem is a storage issue and not a production issue.

With the obvious role of Perilipin and ADFP in the regulation of lipid stores in the body, it was hypothesized that variations in these genes would produce noticeable phenotype differences in adipose storage at the population level. It was also hypothesized that since ADFP is involved in foam cell formation and the regulation of triglycerides within these macrophages, that SNPs in the ADFP genome would produce a noticeable effect on serum lipid levels at the population level. The analysis consisted of investigating whether a genotype effect existed; whether a certain genotype was

associated with significantly different body composition (%BF) or serum lipid parameter and the results can be found in **table 3.12**.

The results for the perilipin analysis show that there was no significant associations found between the SNPs analyzed and body composition or for any serum lipid parameter (after accounting for gender, age) (**Table 3.12**). One of the most consistent and striking observations regarding perilipin gene polymorphisms is the effect of gender on associated metabolic phenotypes with most associations being found mostly in women. Our analysis corrected for any effect of gender on the reported results. As well, variations in body fat and serum lipids are highly correlated with age. As such we accounted for variations due to age in our analysis.

Perilipin has been associated with human disease in numerous studies (**Table 4.1**) [95-98, 100, 193-195]. The majority of these studies have to do with the association of perilipins with obesity and obesity associated diseases. One of the first differences in these studies and the work presented in this thesis is that these other studies used BMI to measure body fat whereas our study used DXA. As has already been discussed in this thesis and published by our lab, BMI misclassifies adiposity status in approximately one-third of women and men compared with DXA [151]. Obviously this would have some effect on the results of a genetic association study if one third of subjects were not classified correctly into obesity groups. Thus it is hard to compare the results of studies that use BMI with studies that use DXA to measure adiposity. In addition, most of these studies were on small population sizes. Furthermore, since candidate gene association studies have been questioned because of non-replication of results, in part due to small

sample size and over-interpretation of data, our study demonstrated a comprehensive study design whereby these factors were minimized.

The other variable investigated was the effect of *PLIN* variations on serum lipid concentrations. There are very few published reports on associations between perilipin and other factors of obesity such as serum lipid levels. This may be in part due to the fact that perilipin may not be involved in the metabolism or regulation of serum lipids as evidence points to its main function as the LSD gatekeeper in adipocytes. However, there is some evidence that suggests associations of variations in the *PLIN* gene with serum lipid levels. A study by Jang *et al.* suggested an association between variations at

Table 4.1: Single nucleotide polymorphisms in *PLIN* and metabolic phenotypes in humans

Polymorphism	Allele	Population	Phenotype	Reference
1243 C/T (rs2304796)	C	Japanese Males	Decreased bone density	[101]
6209 T/C (rs2289487)	C	White and Spanish Females	Decreased obesity risk	[96]
		White Females	Increased risk of DM2	[193]
10171 A/T (rs8179043)	T	White Females	Increased risk of DM2	[193]
11482 G/A (rs894160)	A	White and Spanish Females	Decreased obesity risk	[96, 193-194]
		Whites	Resistance to weight loss	[95]
		Koreans with type DM2	Rosiglitazone dependent weight gain	[196]
		Asian and White Females	Increased risk of DM2	[100]
13041A/G (rs2304795)	A	White Females	Increased obesity risk	[98]
14995 A/T (rs1052700)	T	White Females	Increased obesity risk	[98]
		Asian Females	Increased obesity risk	[97]
		Asian Females	Increased risk of DM2	[100]

Adapted from Bickel, 2009.[71] DM2= Diabetes Mellitus Type 2

the perilipin locus and changes in serum free fatty acids and body fat after completion of a prescribed weight loss program [94]. They found that GG homozygotes of the SNP 10076C_G experienced a significantly higher reduction of serum FFA compared with CC homozygotes after adjustment for age, gender and BMI. In addition, G carriers at SNP 11482G_A or A carriers at SNP 14995A_T also had reductions in FFA concentrations after a prescribed weight loss program. These results are not unexpected as one would predict a reduction in FFA levels secondary to increased activity and a decrease in oral intake as was prescribed in the weight loss program in this study. However, one interesting finding was that subjects homozygous for the A allele at SNP 11482 or SNP 14995 showed an increase in serum FFA after completion of the prescribed weight loss program. These results suggest that perilipin may do more than modify the fat content of adipocytes and may play a significant role in the determination of serum lipid concentrations.

Our study did not find any association between variations in the perilipin gene and serum lipids. Although this may seem in contrast to the results of Jang *et al.*, their study differed from our study in a few ways. First, the analysis was carried out using a much smaller population (177) than our study (1200). Second, the subjects were of different ethnicity (Korean) than the subjects used in our study (white). Third, Jang studied different SNPs in the perilipin locus than us. Fourth, they used BMI to measure subjects where we used DXA, however this would not be thought to impact on their results involving serum lipids. Finally, Jang's group also performed their study on subjects who had just finished a prescribed weight loss program. Our subjects were not under any such guidelines or manipulations.

Another comparable study to our research is work published by Qi *et al.* [98].

This study is more comparable as it used DXA to measure body fat, had a fairly large sample size (734) and used a mostly white population. The main difference between our study and that of Qi *et al.* is that they used different SNPs than us. The SNPs Qi *et al.* used have been well established as being associated with body composition in the literature. Our focus was to do novel research on the *PLIN* gene, and thus we chose SNPs not previously reported in obesity research. Qi's main finding was that two SNPs (13041A_G, and 14995A_T) were significantly associated with %BF in women. Qi also found that women who were homozygous for the minor allele A in the *PLIN* SNP 11482, another well reported *PLIN* SNP, appeared to have higher LDL-C measurements. This is in agreement to what Jang's group found for this SNP. In addition, this research agrees with earlier research by Mottagui-Tabar *et al.* in which the A homozygote in this SNP (*PLIN* 11482) was associated with increased adipose lipolysis rates and decreased adipocyte perilipin concentrations in white women [194]. Both basal and stimulated lipolysis rates were increased two to four fold and perilipin protein levels were reduced by up to 80%. It was hypothesized by Qi *et al.* that the elevated fatty acid in circulation secondary to the increased levels of lipolysis would increase the rate of FFA influx into the liver resulting in altered lipid metabolism and dyslipidemia. This work however, has not been duplicated. Our results did not support either of these findings as the SNPs we investigated in *PLIN* in the Newfoundland population did not show any significant associations with either body fat or serum lipids.

In summary, since no significant difference existed between perilipin genotype variations for any SNP investigated and body composition (after accounting for gender

and age) or for any serum lipid parameter (after accounting for gender, age, and %BF), the results suggest no relationship exists. Thus, the results of the present study do not support a significant role for genetic variations within the perilipin gene in the regulation of body composition and serum lipid profiles in the NL population.

The results for ADFP analysis showed that SNP rs35629534 did not have any significant associations with any of the body fat or serum lipid parameters analyzed. SNP rs3824369 did not show any significant associations with serum HDL-C or serum TAG levels (**Table 3.12**). Our results did show a significant association between SNP rs3824369 and %BF, serum cholesterol and serum LDL-c after controlling for the effects of gender and age (**Table 3.12**). Carriers of the major allele of rs3824369 had significantly higher fasting levels of LDL-c and significantly higher fasting levels of serum cholesterol compared to homozygote carriers of the minor allele (**Table 3.13**, **Table 3.14**). In addition, carriers of the major allele of SNP rs3824369 had significantly higher %BF compared to homozygote carriers of the minor allele (**Table 3.15**). To our knowledge this is the first report of an association between a SNP in *ADFP* and variations in body fat or serum lipids.

Although these results have never been reported before *in vivo*, there is evidence which may explain this association. First is the fact that ADFP is expressed in all cell types [107]. This helps understand that the actions of LSD proteins other than perilipin are not limited to the storage of fat in adipocytes within fat tissue. The role of ADFP in the uptake and release of TAG and cholesterol in LSDs found in other cell types may be crucial to the understanding of dyslipidemia. ADFP upregulation in response to lipid

loading in human monocytes may also offer an explanation to increased serum lipid levels found in our study [117].

Cell based studies produced the first evidence in support of a link between ADRP and lipoprotein synthesis. Magnusson's work showed that ADRP overexpression in primary rat hepatocytes resulted in increased storage of TAG into LSDs and decreased secretion of VLDL [197]. They also showed that ADRP knockout hepatocytes had decreased storage of TAG increased VLDL secretion and increased beta oxidation, supposedly to handle the excess FFA.

As ADFP is involved with lipid metabolism and expressed ubiquitously in the body it makes sense that a significant association was found in our population. However, caution should be taken as the sample size that contained the minor allele for SNP rs3824369 was quite small and thus the results may be erroneous for just this fact. However, these results will serve as strong preliminary findings for further investigation in other studies.

Chapter 5: Concluding remarks

Obesity continues to be one of the most important global health threats for both adults and children. Over one third of the world's population is overweight or obese with these estimates continuing to grow every year. Kelly *et al.* estimates that this number will increase upwards of 60% by the year 2030 [2]. In Canada, this problem is as severe if not worse with over 36% either overweight or obese [5]. The province of Newfoundland and Labrador has one of the highest rates of obesity in Canada, especially in women [6-7].

For many years the definition of obesity has been largely based on measurements of BMI. Our study showed that BMI misclassified 42% of women and 32.5% of men into an incorrect obesity category. The misclassification was gender specific and influenced by age, with the largest discrepancy observed in women under 40 years old.

BMI is a very useful tool as it is inexpensive, non-invasive and easy to perform. We do not recommend for individuals to stop using BMI in everyday use as it is very useful tool to follow ones weight loss or to give a broad overview on ones potential obesity status. However, when BMI is used for research or clinical reasons, care should be taken as there may be large inaccuracies in the results and a more specific measure of body fat should be used.

Calcium has long been thought to be involved in the etiology of some forms of dyslipidemia. However this relationship has never fully been investigated while controlling for confounding factors such as age, gender, medication use, disease status, menopause, parathyroid hormone and 23-OH-vitamin D status. Our study performed such an experiment and determined that abnormalities in serum lipid profiles were

significantly correlated with altered serum Ca^{++} levels independent of the aforementioned confounding factors further strengthening the possibility of a direct relationship between the two.

The candidate gene association study followed up on a well accepted theory that adiposity is closely related to the genetics of how lipids are stored and released from fat stores in the body. Perilipin and its close cousin adipophilin are unique in that they both coat lipid storage droplets. Perilipin is restricted to adipose tissue and steroidogenic cells and adipophilin is expressed ubiquitously in all cell types. We have demonstrated that genetic variants within the perilipin gene do not appear to have a significant association with obesity phenotypes in the Newfoundland and Labrador population. We also demonstrated that genetic variants within the adipophilin gene did appear to have a significant association with obesity phenotypes in the Newfoundland and Labrador population. Moreover, percentage body fat measure by DXA and serum LDL-c and cholesterol were positively associated with a single nucleotide polymorphism (rs3824369) in the adipophilin gene. Subjects who were homozygous for the major allele of the polymorphism had significantly higher levels of body fat, LDL-c and serum cholesterol than subjects who were homozygous for the minor allele. This finding was independent of gender and age. Although the number of homozygotes for the minor allele was quite small, if this result holds true it will be the first such publication linking a SNP in adipophilin with obesity phenotypes such as these.

Chapter 6: Limitations of the present research

6.1: Comparison of the classification of obesity by BMI versus dual-energy x-ray absorptiometry.

1. Although 1691 subjects is a reasonable number to perform analyses on, replication of our results with another cohort would be preferable.
2. Even though recruitment of men was difficult, we did manage to include 370 men in our study. However, a larger cohort of men would be preferred to increase the power in our findings.
3. Due to population constraints, our subjects were limited to white Canadians of European decent. In order for our results to have a more worldwide application, subjects of other races and nationalities would need to be studied in large cohorts such as ours.

6.2: Abnormality of serum lipids are independently associated with increased serum calcium levels in the adult Newfoundland population.

1. Although 1269 subjects is a reasonable number to perform analyses on, replication of our results with another cohort would be preferable.
2. Even though recruitment of men was difficult, we did manage to include 267 men in our study. However, a larger cohort of men would be preferred to increase the power in our findings.
3. Due to population constraints, our subjects were limited to white Canadians of European decent. In order for our results to have a more worldwide

application, subjects of other races and nationalities would need to be studied in large cohorts such as ours.

6.3: Genetic association study – roles of Perilipin and Adipophilin on obesity phenotypes.

1. Although 1691 subjects is a reasonable number to perform association analyses, replication of our results with another cohort would be preferable.
2. Even though recruitment of men was difficult, we did manage to include 370 men in our study. However, a larger cohort of men would be preferred to increase the power in our findings.
3. Due to population constraints, our subjects were limited to white Canadians of European decent. In order for our results to have a more worldwide application, subjects of other races and nationalities would need to be studied in large cohorts such as ours.
4. Although the location of the SNPs chosen was well thought out as to be possible important locations within the locus of each of perilipin and adipophilin, the low number of SNPs genotyped decreases the power of this study. Increasing the number of SNPs studied within each gene to 6 or more would bring this research up to current standards for association studied on candidate genes. Not only would this increase the validity of our study but would also help to strengthen any findings that we make. Unfortunately, time and funding were not available for us to continue the research in this direction.

7.1: Comparison of the classification of obesity by BMI versus dual-energy x-ray absorptiometry.

1. This research was originally planned to be used to create new criteria for BMI to help decrease the discrepancy found between BMI and more accurate anthropometric tools. It is hoped that one day it will be used for this purpose.

7.2: Abnormality of serum lipids are independently associated with increased serum calcium levels in the adult Newfoundland population.

1. It is planned that these results will be correlated with dietary intake for further understanding into serum calcium effects on serum lipids.

7.3: Genetic association study – roles of Perilipin and Adipophilin on obesity phenotypes.

1. Since the completion of this original research, more than 700 subjects have been recruited into the CODING study. The remaining subjects will be genotyped.
2. In addition to genotyping the new recruited subjects, more SNPs will need to be genotyped within each gene to increase the power of the findings. These new SNPs will be strategically chosen based on their location, linkage

~~disequilibrium with the SNPs already genotyped and new SNPs not yet~~
genotyped and as well based on published results in the literature.

3. Information on dietary intake of fat has been collected from study participants and analyzed using the Willet Food Frequent Questionnaires and the Nutribase clinical nutritional software. Combining this information with fasting lipid profiles already collected from study subjects and the results from the SNPs genotyping analysis will help provide understanding of the interactions between genetic variations in LSD associated genes and dietary fat consumption on serum cholesterol LDL-c, HDL-c and TAGs. Preliminary data has been produced and results are encouraging.

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